

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07K 15/00, C07H 15/12 C12N 15/00, G01N 33/53	A1	(11) International Publication Number: WO 92/16558 (43) International Publication Date: 1 October 1992 (01.10.92)
(21) International Application Number: PCT/US92/02109 (22) International Filing Date: 12 March 1992 (12.03.92) (30) Priority data: 667,957 12 March 1991 (12.03.91) US (71) Applicant: THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US). (72) Inventors: ALTIERI, Dario, C. ; 8870 Via La Jolla Drive, #305, La Jolla, CA 92037 (US). EDGINGTON, Thomas, S. ; 2362 Avenida de la Playa, La Jolla, CA 92037 (US).		(74) Agents: FITTING, Thomas et al.; The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037 (US). (81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (Euro- pean patent), MC (European patent), NL (European pa- tent), SE (European patent). Published <i>With international search report.</i>
(54) Title: CELL SURFACE RECEPTORS HOMOLOGOUS TO COAGULATION FACTORS V AND VIII (57) Abstract A new class of cellular receptors extensively homologous but not identical to coagulation factors V and VIII is identified. Specific amino acid residue sequences of the receptor are described. Antibody compositions capable of immunoreacting with polypeptides containing the identified amino acid residue sequences and related diagnostic protocols are also described. The receptors are demonstrated to bind coagulation factor Xa which binding is inhibited by monoclonal antibodies to the receptors.		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MI	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

CELL SURFACE RECEPTORS HOMOLOGOUS
TO COAGULATION FACTORS V AND VIII

Description

5 Technical Field

 The present invention relates to a new class of extracellular receptor molecules homologous to human coagulation factors V and VIII, and activated forms thereof. Amino acid residue sequences of the
10 receptors are described, as are antibodies immunoreactive with the receptor molecules and associated diagnostic kits.

Background

 The assembly of proteolytic activities on
15 cellular surfaces initiates a variety of essential biologic responses. Specific high affinity receptors coordinate such interactions, protect the protease from inactivation by ubiquitous extracellular inhibitors, and provide optimal spatial alignment for
20 the catalytic efficiency of the enzyme. The regulated association of coagulation and fibrinolytic proteins with a variety of cells may well exemplify these mechanisms of specialized protease-cell interactions [Miles, L. A., et al., Fibrinolysis, 2:61 (1988);
25 Morrissey, et al., Cell, 50:129 (1987); Nesheim, M. E., et al., J. Biol. Chem., 254:10952 (1979)].

 It has become increasingly clear, however, that the same enzymes that participate in blood coagulation and fibrinolysis also mediate additional and disparate
30 biologic functions. With surprising analogies with the mechanisms of hormone-mediated growth factor activity, thrombin exerts a potent mitogenic effect on various cell types in a reaction exquisitely coordinated by specific cellular receptors [Chen, L.
35 B., et al., Proc. Natl. Acad. Sci. USA, 72:131 (1975);

Glenn, K. C., et al., Nature, 278:711 (1979); Baker, J. B., et al., Nature, 278:743 (1979)]. Similarly, the delicate balance between mitogenesis, malignant transformation, protooncogene expression, and cell differentiation has been shown to be profoundly influenced by protease activity [Unkeless, J. C., et al., J. Exp. Med., 137:85 (1973); Sullivan, L. M., et al., Cell, 45:905 (1986); and Fenner, F. et al. in The Orthopoxviruses Academic Press (San Diego) (1989)].

Various immune-inflammatory reactions are no exceptions. The binding of urokinase as well as of thrombin to their complementary cellular receptors produces a potent chemotactic reaction with local accumulation of neutrophils and monocytes in vivo [Bowie, M.D.P., et al., J. Immunol., 139:169 (1987); Bar-Shavit, R., et al., Science, 220:728 (1983)]. Moreover, synthetic protease inhibitors have been shown to decrease or abolish NK- and CTL-mediated target cell lysis, as well as monocyte synthesis and release of TNF- α [Redelman, D., et al., J. Immunol., 124:870 (1980); Chang, T. W., et al., J. Immunol., 124:1028 (1980); Suffys, P., et al., Eur. J. Biochem., 178:257 (1988); Scuderi, P., J. Immunol., 143:168 (1989)].

This concept of a more direct participation of proteases in specific cellular immune effector functions has been recently further enforced by the identification of a family of related serine proteases in cytotoxic NK and CTL clones [Masson, D., et al., Cell, 49:679 (1987)]. These serine proteases, termed granzymes [Jenne, D., et al., Proc. Natl. Acad. Sci. USA, 85:4814 (1988)], are compartmentalized in subcellular granules together with the pore-forming protein perforin and are locally released during the polarized exocytosis associated with the formation of

endothelial:T cell conjugates [Masson, D., et al., J. Biol. Chem., 260:9069 (1985); Pasternack, M. S., et al., Nature, 322:740.12 (1986); Podack, E. R., et al., J. Exp. Med., 160:695 (1984)].

5 As revealed by molecular cloning, several granzymes share a remarkable degree of homology with other serine proteases involved in coagulation and fibrinolysis, and particularly with the plasma
10 coagulation proteases factors IXa and Xa [Jenne, D., et al., Proc. Natl. Acad. Sci. USA, 85:4814 (1988); Gershenfeld, H. K., et al., Science, 232:854 (1986); Jenne, D., et al., J. Immunol., 140:318 (1988); Lobe, C. G., et al., Science, 232:858 (1986); Gershenfeld, H. K., et al., Proc. Natl. Acad. Sci. USA, 85:1184
15 (1988)]. While compelling evidence has accumulated for a direct role of perforin in target cell injury [Masson, D., et al., J. Biol. Chem., 260:9069 (1985); Duke, R. C., et al., J. Exp. Med., 170:1451 (1989)], the participation and mechanistic role of the
20 granzymes or other serine proteases in the lytic process remains unclear [Dennert, G., et al., Proc. Natl. Acad. Sci. USA, 84:5004 (1987)].

25 Elucidation of the structures and reactive properties of granzymes and other serine proteases likely would lead to a better understanding of the biologic roles played by these entities. Such knowledge could afford improved diagnostic probes for abnormal cellular conditions and new therapeutic tools that reverse associated diseases.

30

Brief Summary of the Invention

The present invention relates to a new class of cellular receptor molecules. The receptor molecules are homologous with certain coagulation cofactors,
35 such as human coagulation factors V and VIII.

Functionally, the receptor molecules bind serine protease ligands, such as the circulating proteins factor Xa, factor IX/IXa and plasmin(ogen). A preferred receptor molecule, referred to herein as EPR-1, is homologous to but different from human factor V and binds factor Xa. Polypeptides containing an amino acid residue sequence homologous to EPR-1 are also contemplated.

A preferred embodiment of the invention, is a purified protein that has a molecular weight of about 78 kDa and an amino acid residue sequence comprising the following amino acid residue sequences:

- (1) Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
- (2) Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
- (3) Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
- (4) Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
- (5) Gly-Val-Pro-Pro-Val-Val-Thr;
- (6) Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
- (7) Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-

Lys-His-Val-Glu-Pro-His-Ala-Thr, wherein Xaa is an unspecified amino acid, which can be in the protein as any naturally-occurring amino acid residue, including a modified or unusual, e.g., glycosylated, residue. The Xaa residue can be identical to or other than another Xaa residue in the molecule. In a further preferred aspect of the invention, the protein immunoreacts with the antibody produced by the hybridoma designated 12H1 (ATCC Accession No. HB 10637). In a still further preferred aspect of the invention, the protein is the EPR-1 protein isolated from the cell line designated MOLT13 #3 (ATCC Accession No. CRL 10638).

Another preferred embodiment of the invention is a polypeptide comprising up to about 600 amino acid residues which includes an amino acid residue sequence

selected from the group of sequences (1)-(7) listed above.

Also contemplated within the invention are a DNA segment that codes for a protein or polypeptide as defined previously and a vector, i.e., self-replicating DNA molecule, including the DNA segment.

In a preferred aspect of the invention, an antibody composition is contemplated which immunoreacts with an instant protein or polypeptide.

A particularly preferred antibody composition is the monoclonal antibody produced by the 12H1 hybridoma. Another preferred antibody composition immunoreacts with a protein isolated from the MOLT13 #3 cell line, which has a molecular weight of about 78 kDa, and includes a before-listed amino acid residue sequence.

A method of assaying for the presence of an instant receptor molecule on a cell surface is also contemplated. The method comprises the steps of:

(a) admixing a cell or cell lysate suspected of expressing the receptor molecule with an antibody composition described before, such as hybridoma 12H1;

(b) maintaining the admixture for a time sufficient to form an immunoreaction product; and

(c) determining the presence of immunoreaction product and thereby detecting presence of the receptor molecule.

Also contemplated is a method of monitoring the response to treatment of a patient having a disease associated with a before-described protein localized on cells in a body sample withdrawn from the patient that is used as a marker for the disease state. The method comprises assaying for the marker using an antibody composition described before, repeating the assay after a course of treatment, and determining the patient's response to treatment as a function of the

amount of that cell surface protein present after treatment. An exemplary disease state monitored is chronic lymphocytic leukemia (CLL).

5 Brief Description of the Drawings

Figure 1 depicts FMF (flow microfluorometry) analysis of monocytes or PMN (polymorphonuclear leucocytes) with anti-EPR-1 mAb 9D4. Suspensions of monocytes were prepared from PBMC (peripheral blood mononuclear cells) by adherence to plastic precoated with autologous serum for 1 hour at 37°C. PMN were isolated by dextran sedimentation. The 1×10^6 cells were stained with 10 µg/ml of mAb 9D4 for 30 minutes in ice, washed, and further incubated with 1/20 dilutions of fluorescein-conjugated goat anti-mouse IgG. Cells were washed and immediately analyzed by FMF. Control mAb was the irrelevant V82A6 and is shown with a dotted line. The abscissa indicates fluorescence intensity on a 4 log scale. The ordinate indicates the cell number.

Figure 2 depicts modulation of EPR-1 expression during T cell activation. Aliquots of freshly isolated PBMC (10×10^6) were polyclonally activated by culture in the presence of 1 µg/ml PHA or Con A for 7 days at 37°C. For antigen-specific expansion, suspensions of PBMC at 10×10^6 were separately cultivated in unidirectional mixed lymphocyte culture (MLC) with irradiated (10,000 rad) Daudi or Raji cells (10×10^6) for 7 days in 5% CO₂ at 37°C. At the end of the incubation, responder T cells were harvested, recovered by centrifugation over Ficoll-Hypaque, washed, stained with aliquots of mAb 12H1 plus fluorescein-conjugated goat anti-mouse IgG + IgM, and analyzed by FMF. The dotted line indicates the background staining with the irrelevant mAb V82A6.

The percentage of 12H1⁺ cells analyzed before each incubation mixture was $6.7 \pm 1.4\%$.

Figure 3 depicts the effects of long term alloreactive stimulation on EPR-1 expression.

5 Unidirectional MLC was set up against irradiated (10,000 rad) Daudi cells and maintained with weekly transfers and 10% T-cell growth factor (TCGF). Aliquots of responder T cells were harvested after various time intervals (Days = 0, 7, 15 and 32),
10 recovered by centrifugation on Ficoll-Hypaque, and analyzed by FCM using anti-EPR-1 mAb 12H1 or the polyclonal antiserum B78.9.

Figure 4 depicts expression of EPR-1 on transformed T cell lines, FCM analysis of a panel of
15 continuous T cell lines in culture with anti-EPR-1 mAb 7G12 was performed as described above for Figure 1. The irrelevant mAb V82A6 was used as a control, and is shown as a dotted line.

20 Detailed Description of the Invention

A. Definitions

Amino Acid Residue Sequence: a series of two or more amino acid residues joined via peptide linkages between adjacent residues to form a peptide or
25 polypeptide. An amino acid residue sequence is conveniently represented by the one or three letter abbreviations for its constituent amino acids. The abbreviations used herein for amino acids are those provided at 37 C.F.R. §1.822(b)(2) and are reproduced
30 in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

ABBREVIATION		AMINO ACID
1-Letter	3-Letter	
	Y	tyrosine
5	G	glycine
	F	phenylalanine
	M	methionine
	A	alanine
	S	serine
10	I	isoleucine
	L	leucine
	T	threonine
	V	valine
	P	proline
15	K	lysine
	H	histidine
	Q	glutamine
	E	glutamic acid
	Z	Glu and/or Gln
20	W	tryptophan
	R	arginine
	D	aspartic acid
	N	asparagine
	B	Asn and/or Asp
25	C	cysteine
	J	Unspecified
	Xaa	

The individual residues comprising an amino acid residue sequence herein may be in the D or L isomeric form as long as the desired functional property is retained by molecule(s) incorporating the amino acid residue sequence. Also, the amino acid residue sequence may include post-translationally modified amino acids, e.g., hydroxylated, glycosylated amino acid residues, or residues linked via disulfide bonds.

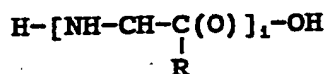
In addition, an amino acid residue sequence can include one or more modified or unusual amino acids, such as those listed in 37 C.F.R. §1.822(b)(4), which are incorporated herein by reference. An amino acid residue sequence can be represented by the abbreviations corresponding to its constituent amino acids in which a hyphen between two adjacent abbreviations indicates a peptide linkage between the corresponding residues.

Antibody: a polypeptide which chemically binds to a haptenic group, i.e., ligand. Antibodies, as used herein, are immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules. Such portions known in the art as Fab, Fab'; F(ab')₂, and F_v are included. Typically, antibodies bind ligands that range in size from about 6 to about 34 Å with association constants in the range of about 10⁴ to 10¹⁰ M⁻¹, and as high as 10¹³ M⁻¹. Antibodies can bind a wide range of ligands, including small molecules such as steroids and prostaglandins, biopolymers such as nucleic acids, proteins and polysaccharides, and synthetic polymers such as polypropylene. An "antibody combining site" is that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) antigen. The term "immunoreact" in its various forms is used herein to refer to binding between an antigenic determinant-containing molecule (antigen) and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof. An "antigenic determinant" is the structural portion of the antigen that is immunologically bound by an antibody combining site. The term is also used interchangeably with "epitope". Antibodies can bind a

single epitope of an antigen (monoclonal) or multiple epitopes (polyclonal).

5 Ligand: a molecule having a structural region that binds specifically to a particular receptor molecule, usually via electrostatic forces and/or hydrogen bonds.

10 Peptide/Polypeptide: a polymer comprising at least two amino acid residues in which adjacent residues are connected by a peptide bond between the alpha-amino group of one residue and the alpha-carbonyl group of an adjacent residue. The primary structure of a polypeptide has a primary amine group at one terminus and a carboxylic acid group at the other terminus of the polymer. Thus, a polypeptide
15 may be represented by the formula:



20 where R is a side chain characteristic of a given amino acid residue and i indicates the number of amino acid residues comprising the polymer which number is two or more. A polypeptide can comprise one or more
25 amino acid residue sequences. Also, a polypeptide in aqueous solution is usually in one or more zwitterionic forms depending on the pH of the solution.

30 Protein: a single polypeptide or set of cross-linked polypeptides comprising more than about 50 amino acid residues. Proteins can have chemical crosslinking, i.e., via disulfide bridges, within the same polypeptide chain or between adjacent
35 polypeptides. Proteins can be glycosylated in which case they are called glycoproteins.

Receptor: a biologically active proteinaceous molecule having a structural region that specifically

binds to (or with) another molecule (ligand).

B. The Polypeptides

5 A polypeptide of the present invention is derived from a new class of cell surface receptors, called effector cell protease receptors (EPRs) because members of the class bind protease ligands and are also found on many types of inflammatory effector cells. The first member of this class (EPR-1) is shown to bind protease ligands of which human factor Xa is prototypic.

15 A polypeptide of the present invention corresponds in amino acid residue sequence to one or more amino acid residue sequence of EPR-1. Moreover, a polypeptide of the invention is shown to have pronounced homologies with the amino acid residue sequence of human coagulation factor V, suggesting a common evolutionary origin of factor V and EPR-1. As factor V includes the residue sequence of factor Va, 20 an instant polypeptide also can have homologies with factor Va. A polypeptide of the invention also can exhibit homology in sequence to a polypeptide portion of factor VIII, as well as to a polypeptide of the murine protein denominated MFG E-8 [Stubbs et al., 25 Proc Natl Acad Sci USA 87:8417 (1990)]. A polypeptide of this invention is nonetheless distinct from factor V, factor VIII or murine MFG E-8.

30 In a preferred embodiment of the invention, the polypeptide is a protein having a molecular weight of about 78 kDa which includes the following amino acid residue sequences:

- (1) Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
- (2) Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
- (3) Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
- 35 (4) Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;

(5) Gly-Val-Pro-Pro-Val-Val-Thr;

(6) Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and

(7) Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-

Lys-His-Val-Glu-Pro-His-Ala-Thr,

5 wherein Xaa is present in the molecule as an
unspecified amino acid residue. The Xaa residue can
be any naturally-occurring amino acid residue
including a modified or unusual residue (cf. 37 C.F.R.
\$1.822(b)(4)). Also, the Xaa residue need not be the
10 same as another Xaa residue in a particular amino acid
residue sequence or in another sequence of the
molecule having an Xaa residue. Characteristically,
the protein has a molecular weight of 78±4 kDa, as
determined by polyacrylamide gel electrophoresis.
15 (The number associated with each amino acid residue
sequence refers to its Sequence I.D. No. as presented
in the sequence listing provided herewith pursuant to
the requirements of 37 C.F.R. §1.821(c)).

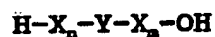
In a further preferred embodiment, the protein is
20 isolated from the cell line designated MOLT13 #3 and
has a molecular weight of about 78 kDa. The MOLT13 #3
cell line was deposited at the American Type Culture
Collection (ATCC) 12301 Parklawn Drive, Rockville,
Maryland, USA 20852. This cell line was deposited on
25 January 11, 1991 and received Accession Number CRL
10638.

The present deposit was made in compliance with
the Budapest Treaty requirements that the duration of
the deposits should be for 30 years from the date of
30 deposit or for 5 years after the last request for the
deposit at the depository or for the enforceable life
of a U.S. patent that matures from this application,
whichever is longer. The cell line will be
replenished should it become non-viable at the
35 depository.

In another embodiment of the invention, the protein is immunoreactive with certain antisera to human factor V as well as with polyclonal antibodies purified from the antisera, e.g., by immunoadsorption on immobilized, purified human factor V. In a further aspect of the invention, the protein immunoreacts with antibodies to human factor VIII. Hence, although the instant EPR protein is not a human factor V or VIII protein per se, it possesses epitopes that are cross-reactive with ligands for certain epitopes of factors V and VIII.

In a further preferred embodiment of the invention, the isolated protein immunoreacts with a small set of antibodies such as those produced by the hybridoma designated 12H1, which was deposited on January 11, 1991, at the ATCC pursuant to the Budapest Treaty as described above. The hybridoma was given the designation ATCC HB 10637.

In another embodiment, a polypeptide of this invention has an amino acid residue sequence represented by the following formula:



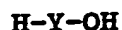
where Y is an amino acid residue sequence selected from the group of amino acid residue sequences (1)-(7) listed above which are present in EPR-1; X_n is absent when $n=0$ and is an N-terminal (leader segment and mature protein) amino acid residue sequence containing up to about 550 residues when $n=1$; and X_m is absent when $m=0$ and is a C-terminal (tail segment) amino acid residue sequence containing up to about 550 residues when $m=1$. The polypeptide comprises up to about 600 amino acid residues.

Preferably, when either X_n or X_m is an amino acid residue sequence, X_n or X_m contains up to about 200 residues, more preferably, up to about 50 residues,

and most preferably, up to about 20 amino acid residues. Typically, X_n and X_m each contain one or more of the before-listed amino acid residue sequences.

5 In a further preferred aspect of the invention, X_n and X_m are selected so that the polypeptide immunoreacts with sera containing antibodies raised to human factor V. More preferably, X_n and X_m are selected so that the polypeptide is a protein having a
10 molecular weight of about 78 kDa. Most preferably, the isolated protein also binds to human factor Xa.

In a still further preferred embodiment, a polypeptide of the invention has the formula:



15 where Y is as defined previously.

A polypeptide of the present invention can be used to generate a variety of useful antibodies by means described herein. The utilities of the polypeptides will be apparent from the discussion
20 provided hereinbelow.

Typically an instant polypeptide is not glycosylated, i.e., it is synthesized either directly by standard peptide synthesis techniques or by procaryotic host expression of a recombinant DNA
25 molecule of the present invention. A eucaryotically produced polypeptide of the present invention is typically glycosylated.

An instant polypeptide can incorporate a variety of changes, such as insertions, deletions, and
30 substitutions of amino acid residues which are either conservative or nonconservative as long as the resulting polypeptide molecule exhibits the desired properties. The "desired properties" as referred to herein include that the polypeptide is immunogenic in
35 a suitable host and able to generate antibodies to the

EPR-1 molecule or a polypeptide homologous to EPR-1, at least in the denatured state as is found in an SDS-PAGE gel, but frequently also in the natural state as expressed on cells. Additionally, the polypeptide is antigenic when expressed on cells or in its denatured state so that antibodies immunoreactive with the EPR-1 molecule also immunoreact with the instant polypeptide.

When an instant polypeptide incorporates conservative substitutions of the sequences corresponding to EPR-1 depicted above, the substituted amino acid residues are replaced by another, biologically similar amino acid residue such that the resulting polypeptide has an amino acid residue sequence that is different from (other than) a sequence of factor V, factor VIII or sequence MFG E-8. Some examples of conservative substitutions include substitution of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another hydrophobic residue. Also, a polar residue such as arginine, glycine, glutamic acid, aspartic acid, glutamine, asparagine, and the like, can be conservatively substituted for another member of this group. Still another aspect of a polypeptide incorporating conservative substitutions occurs when a substituted amino acid residue replaces an unsubstituted parent amino acid residue. Examples of substituted amino acids may be found at 37 C.F.R. §1.822(b)(4), which species are incorporated herein by reference. When the polypeptide has an amino acid residue sequence that corresponds to the sequence of EPR-1 but has one or more conservative substitutions, preferably no more than about 40%, and more preferably no more than about 20%, of the amino acid residues of the native protein are substituted.

A polypeptide of the present invention can be synthesized by any of the peptide synthetic techniques known to those skilled in the art. A summary of some of the techniques available can be found in J.M. Stuard and J. D. Young, "Solid Phase Peptide Synthesis", W. H. Freeman, Co., San Francisco (1969), J. Meinhofer, "Hormonal Proteins and Peptides" Vol. 2, pp. 46, Academic Press (New York) 1983, and U.S. Patent No. 4,631,211, which description is incorporated herein by reference. When a polypeptide desired for use in the present invention is relatively short (less than about 50 amino acid residues in length) direct peptide synthetic techniques are generally favored, usually by employing a solid phase technique such as that of Merrifield [Merrifield JACS, 85:2149 (1963)].

An instant polypeptide can also be synthesized by recombinant DNA techniques. Such recombinant techniques are favored especially when the desired polypeptide is relatively long (greater than about 50 amino acids residues in length). When recombinant DNA techniques are employed to prepare an instant polypeptide, a DNA segment coding for the desired polypeptide is incorporated into a preselected vector that is subsequently expressed in a suitable host. The expressed polypeptide, containing at least one of amino acid residue sequences (1)-(7) corresponding to EPR-1 identified above, is preferably purified by a routine method such as gel electrophoresis, immunosorbent chromatography, and the like.

C. DNA Segments

When recombinant DNA techniques are employed to prepare a polypeptide of the present invention, a DNA segment encoding the polypeptide is used. A DNA

segment contemplated within the invention is operatively linked to a vector that is subsequently expressed in a suitable host. The segment is "operatively linked" to the vector as used herein when it is ligated (covalently bonded) thereto, as is well known. Also contemplated is an RNA segment equivalent to an instant DNA segment.

The present DNA segment is a molecule that can be readily synthesized by chemical techniques, e.g., by the well-known phosphotriester method [Matteucci et al., JACS, 103:3185 (1981)]. By chemically synthesizing the DNA segments, any desired substitution, insertion or deletion of an amino acid residue or sequence from a template polypeptide, e.g., the native protein, can be readily provided by simply making the corresponding changes in the nucleotide sequence of the DNA segment.

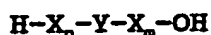
Whenever an RNA segment coding for the instant polypeptide is used, the RNA molecule including the polypeptide coding segment is transcribed into complementary DNA (cDNA) via a reverse transcriptase. The cDNA molecule can then be transcribed and translated as described herein to generate a desired polypeptide.

In a preferred aspect of the invention, a DNA nucleotide sequence (segment) coding for at least one of the amino acid residue sequences (1)-(7) of EPR-1 identified above is operatively linked to a larger DNA molecule. The resultant DNA molecule is then transformed in a suitable host and expressed therein.

The DNA segment coding for an amino acid residue sequence (1)-(7) listed above can be provided with start and stop codons or one or both of the start and stop codons can be provided by the larger DNA molecule, e.g., vector, operatively linked to the DNA

segment so that only the corresponding polypeptide is generated. Alternatively, a nucleotide sequence coding for additional amino acid residues can be provided at the 3' and/or 5' ends of the DNA segment so that a larger polypeptide is expressed having an amino acid residue sequence at either or both of its N-terminal and C-terminal ends in addition to an amino acid residue sequence (1)-(7) listed above of the EPR-1 molecule.

10 A DNA molecule of the invention can encode a polypeptide having an amino acid residue sequence represented by the formula:



where X_n , Y, and X_m are as defined previously.

15 Preferably, the DNA segment encodes a polypeptide up to about 600 amino acid residues in length. When the DNA segment encodes a polypeptide having either X_n or X_m as an amino acid residue sequence, X_n or X_m contains up to about 200 residues, more preferably up to about 20 residues, and most preferably, up to about 20 amino acid residues. Typically, one or both of the flanking regions to the DNA segment encoding the Y sequence of the polypeptide encodes one or more of the amino acid residue sequences (1)-(7) listed above.

25 An instant DNA molecule can also be produced by enzymatic techniques. Thus, restriction enzymes which cleave DNA molecules at predefined recognition sequences can be used to isolate DNA fragments from larger DNA molecules containing the desired DNA segments such as the DNA (or RNA) that codes for the EPR-1 protein. Typically, DNA fragments produced in this manner will have cohesive, "overhanging" termini, in which single-stranded nucleotide sequences extend beyond the double-stranded portion of the molecule.

30

35 The presence of such cohesive termini is generally

preferred over blunt-ended DNA molecules. The isolated fragments containing the desired coding sequence can then be ligated (cloned) into a suitable vector for amplification and expression.

5 Additionally, an instant DNA segment can be generated by polymerase chain reaction (PCR) techniques, which amplify targeted DNA segments of a template nucleic acid. In PCR, a specific polynucleic acid target is transcribed by a reaction in which a
10 primer molecule complementary to a particular section of a nucleic acid template is used to form an extension product of the primer including a nucleic acid region complementary to the target. After
15 separation of template and extended primer, each primer extension product acts as a template and specifically anneals with a complementary primer molecule. The resulting primed template acts as a
20 substrate for further extension reactions. These steps are repeated, preferably using an automated cycling procedure, thereby exponentially amplifying the initial polynucleic acid target to which the
25 primer hybridizes. Procedures for conducting PCR have been extensively described, see, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, which descriptions are incorporated herein by reference.

 In using PCR technology herein, a DNA primer molecule coding for one or more of the before-
enumerated amino acid residue sequences (1)-(7) is preferably utilized. However, additional nucleotide
30 sequences can be revealed by cloning the cDNA or genomic DNA encoding EPR-1 and smaller amino acid residue sequences thereof. A DNA probe molecule encoding an EPR-1 amino acid residue sequence having
minimal homology to either of factors V or VIII is
35 usually employed in order to maximize specificity of

hybridization with DNA or RNA encoding the targeted
EPR-1 amino acid residue sequence and to minimize
hybridization with DNA or RNA coding for either factor
V, factor VIII, or homologous, nontargeted molecules.
5 However, such "rule of thumb" regarding the homology
of a probe molecule is not critical in identifying a
suitable probe, and indeed, may not be appropriate at
all, as when examined cells do not express factors V
or VIII. The use of mixed, redundant primers that
10 encode a targeted amino acid residue sequence
utilizing different codons for the same amino acid
residue is of course contemplated.

D. Vectors

15 Also contemplated within the present invention is
a vector that can be operatively linked to an instant
DNA segment to provide a self-replicating recombinant
DNA molecule that encodes an instant polypeptide,
preferably expressing the EPR-1 protein itself. The
20 recombinant molecule can be used to transform suitable
host cells so that the host cells express the desired
polypeptide. Hence, the DNA molecule can be regarded
as self-replicating.

The choice of vector to which a DNA segment of
25 the present invention is operatively linked depends,
as is well known in the art, on the functional
properties desired, e.g., efficiency of expression,
the transformation host cell, and the like. However,
a vector of the present invention is at least capable
30 of directing the replication, and preferably also
expression, of a DNA segment coding for an instant
polypeptide.

Preferably, a chosen vector includes a
procaryotic replicon, i.e., a DNA sequence, having the
35 ability to direct autonomous replication and

maintenance of the recombinant DNA molecule extrachromosomally in a procaryotic host cell transformed therewith. Such replicons are well known in the art. In addition, a vector that includes a
5 procaryotic replicon preferably also includes a drug resistance gene so that hosts transformed with a vector can be readily screened. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

10 Vectors that include a procaryotic replicon preferably include a procaryotic promoter capable of directing the transcription of the instant polypeptide genes. A promoter is an expression control element formed by a DNA sequence that promotes binding of RNA
15 polymerase and transcription of single-stranded DNA into messenger RNA (mRNA) molecules. Promoter sequences compatible with bacterial hosts, such as a tac promoter, are typically provided in plasmid vectors having convenient restriction sites for
20 insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA) and pPL and pKK223 available from Pharmacia (Piscataway, NJ).

25 Expression vectors compatible with eucaryotic cells, preferably those compatible with vertebrate cells, can also be used to form a recombinant DNA molecule described before. Eucaryotic cell expression vectors are well known in the art and are available
30 from several commercial sources. Typically, such vectors are provided with convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1pML2d (International Biotechnologies, Inc.), and
35 pTDT1 (ATCC, #31255). A preferred drug resistance

marker for use in vectors compatible with eucaryotic cells is the neomycin phosphotransferase (neo) gene. [Southern et al., J. Mol. Appl. Genet., 1:327-341 (1982)].

5 Retroviral expression vectors capable of generating the recombinant DNA of the present invention are also contemplated. The construction and use of retroviral vectors for generating desired DNA molecules have been described by Sorge, et al., Mol.
10 Cell. Biol., 4:1730-37 (1984).

 A number of methods are available to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted
15 and to the vector DNA. The vector and DNA segment are then allowed to hybridize by hydrogen bonding between the complementary homopolymer tails to form recombinant duplex DNA molecules.

 Alternatively, synthetic linkers containing one
20 or more restriction sites can be used to join the DNA segment to vectors. When the DNA segment is generated by endonuclease restriction digestion, as described earlier, it is treated with bacteriophage T4 DNA polymerase of E. coli DNA polymerase I which removes
25 protruding 3' single-stranded termini and fills in recessed 3' ends. Blunt-ended DNA segments are thereby generated.

 Blunt-ended DNA segments are incubated with a large molar excess of linker molecules in the presence
30 of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments bonded at their ends to linker sequences having restriction sites therein. The restriction
35 sites of these DNA segments are then cleaved with the

appropriate restriction enzyme and the segments ligated to an expression vector having termini compatible with those of the cleaved DNA segment. Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies, Inc. (New Haven, CT).

E. Transformation of Hosts

The present invention also relates to host cells transformed with a recombinant DNA molecule of the present invention. The host cell can be either procaryotic or eucaryotic. Preferred procaryotic host cells are strains of E. coli, e.g., the E. coli strain DH5 available from Bethesda Research Laboratories, Inc., Bethesda, MD. Preferred eucaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from mouse, rat, monkey or human fibroblastic cell line. Preferred eucaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61 and NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658. Transformation of appropriate cell hosts with a recombinant DNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with retroviral vectors containing RNA encoding the instant polypeptides and a reverse transcriptase, see, e.g., Sorge et al., Mol. Cell. Biol., 4:1730-37 (1984). Successfully transformed cells, i.e., those

containing a recombinant DNA molecule of the present invention, can be identified by well known techniques. For example, transformed cells can be cloned to produce monoclonal colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the desired DNA segment using a method such as that described by Southern, J. Mol. Biol., 98:503 (1975).

In addition to directly assaying for the presence of the desired DNA segment, successful transformation can be confirmed by well known immunological methods when the DNA directs expression of the polypeptides of the present invention. Samples of cells suspected of being transformed are harvested and assayed for antigenicity by antibodies that specifically bind to the instant polypeptides.

In addition to the transformed host cells themselves, also contemplated by the present invention are cultures of those cells. Nutrient media useful for culturing transformed host cells are well known in the art and can be obtained from several commercial sources. In embodiments wherein the host cell is mammalian a "serum-free" medium is preferably used.

Methods for recovering an expressed protein from a culture are well known in the art. For instance, gel filtration, gel chromatography, ultrafiltration, electrophoresis, ion exchange, affinity chromatography and related techniques can be used to isolate the expressed proteins found in the culture. In addition, immunochemical methods, such as immunoaffinity, immunoadsorption, and the like, can be performed using well known methods, as exemplified by the methods described herein.

F. Antibody Compositions

Also contemplated within the present invention is an antibody composition that immunoreacts with an instant polypeptide. An antibody composition immunoreacts with the polypeptide either associated with cellular surfaces or free from cellular structures. Thus, an antibody composition binds to one or more epitopes presented by the polypeptide on the exterior surface of cells or to the epitopes of cell-free polypeptides.

A preferred antibody composition of the invention immunoreacts with an EPR-1 protein molecule presented on the cell surface or free of cellular components as when the EPR-1 molecule is isolated upon lysis of cells carrying the molecule. Particularly preferred antibody compositions in this regard are the monoclonal antibodies (mAbs) designated 7G12, 9D4, and 12H1. Such mAbs are obtained as described herein and in Altieri et al., J. Biol. Chem., 264(5):2969 (1989) and Altieri et al., J. Immun. 145:246 (1990). Of course, polyclonal antibodies are also contemplated.

Briefly, a preferred antibody composition is generated by immunizing mice with human factor V, factor VIII or a polypeptide of this invention. The antibodies generated are screened for binding affinity for a polypeptide of the instant invention, such as EPR-1. Isolated EPR-1 or EPR-1 on washed lymphocytes free of factor V or factor VIII can be used for screening the antibodies.

Typically the instant mAbs immunoreact both with factor V and with the target polypeptide of this invention having homology with factor V. However, when a polypeptide homologous but not identical to factor V is used to obtain the instant mAbs, the mAbs preferably immunoreact with the target polypeptide but not with the blood coagulation factor. The mAbs can

also immunoreact with factor VIII proteins similar to the reaction with factor V.

Since the antibodies of the present invention can bind to receptors for coagulation factor Xa, they can be used to competitively inhibit factor Xa from binding to sites on cellular surfaces. Thus, the protease activity of factor Xa in the region of the cell surfaces can be curtailed. Methods for inhibiting such binding are well known to those skilled in the art.

A preferred antibody composition as contemplated herein is typically produced by immunizing a mammal with an inoculum containing human factor V or a polypeptide of the present invention, thereby inducing in the mammal antibody molecules having the appropriate immunospecificity for the immunogenic polypeptide. The antibody molecules are then collected from the mammal, screened and purified to the extent desired by well known techniques such as, for example, by immunoaffinity for the immunogen immobilized on a solid support. The antibody composition so produced can be used inter alia, in the diagnostic methods and systems of the present invention to detect expression of the instant polypeptides on the surface of cells, e.g., leukocytes of patients with chronic lymphocytic leukemia (CLL).

A monoclonal antibody composition (mAb) is also contemplated by the present invention, as noted before. The phrase "monoclonal antibody composition" in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with a particular antigen. The instant mAb composition thus typically displays a single binding affinity for any antigen with which it

immunoreacts. However, a given monoclonal antibody composition may contain antibody molecules having two different antibody combining sites, each immunospecific for a different antigenic determinant, i.e., a bispecific monoclonal antibody.

An instant mAb is typically composed of antibodies produced by clones of a single cell called a hybridoma that secretes (produces) but one kind of antibody molecule. The hybridoma cell is formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. Such antibodies were first described by Kohler and Milstein, Nature 256:495-497 (1975). A particularly preferred hybridoma is designated 12H1 (ATCC Accession No. HB 10637).

A monoclonal antibody can also be produced by methods well known to those skilled in the art of producing chimeric antibodies. Those methods include isolating, manipulating, and expressing the nucleic acid that codes for all or part of an immunoglobulin variable region including both the portion of the variable region comprising the variable region of immunoglobulin light chain and the portion of the variable region comprising the variable region of immunoglobulin heavy chain. Methods for isolating, manipulating, and expressing the variable region coding nucleic acid in procaryotic and eucaryotic hosts are disclosed in Robinson et al., PCT Publication No. WO 89/0099; Winter et al., European Patent Publication No. 0239400; Reading, U.S. Patent No. 4,714,681; Cabilly et al., European Patent Publication No. 0125023; Sorge et al., Mol. Cell Biol., 4:1730-1737 (1984); Beher et al., Science, 240:1041-1043 (1988); Skerra et al., Science, 240:1030-1041 (1988); and Orlandi et al., Proc. Natl.

Acad. Sci., U.S.A., 86: 3833-3837 (1989). Typically the nucleic acid codes for all or part of an immunoglobulin variable region that binds a preselected antigen (ligand). Sources of such nucleic acid are well known to one skilled in the art and, for example, can be obtained from a hybridoma producing a monoclonal antibody that binds the preselected antigen, or the preselected antigen can be used to screen an expression library coding for a plurality of immunoglobulin variable regions, thus isolating the nucleic acid.

The present invention contemplates a method of forming a monoclonal antibody molecule that immunoreacts with a polypeptide of the present invention, and optionally a factor V or VIII protein obtained from a mammal. The method comprises the steps of:

(a) Immunizing an animal with a polypeptide of this invention or a protein homologous thereto, such as a factor V or VIII protein. Conveniently, the immunogen is a protein taken directly from a subject animal species. However, the antigen can also be linked to a carrier protein such as keyhole limpet hemocyanin, particularly when the antigen is small, such as a polypeptide consisting essentially of an amino acid residue sequence (1)-(7) listed above. The immunization is typically performed by administering the sample to an immunologically competent mammal in an immunologically effective amount, i.e., an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained for a time period sufficient for the mammal to produce cells secreting antibody molecules that immunoreact with the immunogen.

(b) A suspension of antibody-producing cells removed from the immunized mammal is then prepared. This is typically accomplished by removing the spleen of the mammal and mechanically separating the individual spleen cells in a physiologically tolerable medium using methods well known in the art.

(c) The suspended antibody-producing cells are treated with a transforming agent capable of producing a transformed ("immortalized") cell line.

Transforming agents and their use to produce immortalized cell lines are well known in the art and include DNA viruses such as Epstein-Barr virus (EBV), simian virus 40 (SV40), polyoma virus and the like, RNA viruses such as Moloney murine leukemia virus (Mo-MuLV), Rous sarcoma virus and the like, myeloma cells such as P3X63-Ag8.653, Sp2/O-Ag14 and the like.

In preferred embodiments, treatment with the transforming agent results in the production of an "immortalized" hybridoma by fusing the suspended spleen cells with mouse myeloma cells from a suitable cell line, e.g., SP-2, by the use of a suitable fusion promoter. The preferred ratio is about 5 spleen cells per myeloma cell in a suspension containing about 10^8 splenocytes. A preferred fusion promoter is polyethylene glycol having an average molecule weight from about 1000 to about 4000 (commercially available as PEG 1000, etc.); however, other fusion promoters known in the art may be employed.

The cell line used should preferably be of the so-called "drug resistant" type, so that unfused myeloma cells will not survive in a selective medium, while hybrids will survive. The most common class is 8-azaguanine resistant cell lines, which lack the enzyme hypoxanthine-guanine phosphoribosyl transferase and hence will not be supported by HAT (hypoxanthine,

aminopterin, and thymidine) medium. It is also generally preferred that the myeloma cell line used be of the so-called "non-secreting" type which does not itself produce any antibody. In certain cases, however, secreting myeloma lines may be preferred.

(d) The transformed cells are then cloned, preferably to monoclonality. The cloning is preferably performed in a tissue culture medium that does not sustain (support) non-transformed cells. When the transformed cells are hybridomas, this is typically performed by diluting and culturing in separate containers the mixture of unfused spleen cells, unfused myeloma cells, and fused cells (hybridomas) in a selective medium which will not sustain the unfused myeloma cells. The cells are cultured in this medium for a time sufficient to allow death of the unfused cells (about one week). The dilution can be a limiting dilution, in which the volume of diluent is statistically calculated to isolate a certain number of cells (e.g., 0.3-0.5) in each separate container (e.g., each well of a microtiter plate). The medium is one (e.g., HAT medium) that does not sustain the drug-resistant (e.g., 8-azaguanine resistant) unfused myeloma cell line.

(e) The tissue culture medium of the cloned transformants is analyzed (immunologically assayed) to detect the presence of antibody molecules that preferentially react with the instant polypeptides or cells bearing the EPR-1 receptor molecule. This is accomplished using well known immunological techniques.

(f) A desired transformant is then selected and grown in an appropriate tissue culture medium for a suitable length of time, followed by recovery

(harvesting) of the desired antibody from the culture supernatant by well known techniques. A suitable medium and length of culturing time are also well known or are readily determined.

5 It is noted that monoclonal antibodies to EPR-1 induced by immunization with factor V are relatively rare. Indeed, only about one percent of the monoclonal antibodies induced by the above method immunoreact with EPR-1.

10 To produce a much greater concentration of slightly less pure monoclonal antibody, the desired hybridoma can be transferred by injection into mice, preferably syngenic or semisyngenic mice. The hybridoma causes formation of antibody-producing
15 tumors after a suitable incubation time, which results in a high concentration of the desired antibody (about 5-20 mg/ml) in the bloodstream and peritoneal exudate (ascites) of the host mouse.

 Media and animals useful for the preparation of
20 these compositions are both well known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium [DMEM; Dulbecco et al., Virology 8:396 (1959)]
25 supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal calf serum. A preferred inbred mouse strain is Balb/c.

 Methods for producing the instant hybridomas which generate (secrete) the antibody molecules of the
30 present invention, are well known in the art and are described further herein. Particularly applicable descriptions of relevant hybridoma technology are presented by Niman et al., Proc. Natl. Acad. Sci. USA, 80:4949-4953 (1983), and by Galfre et al., Meth.
35 Enzymol., 73:3-46 (1981), which descriptions are

incorporated herein by reference.

A further preferred method for forming the instant antibody compositions involves the generation of libraries of Fab molecules using the method of Huse et al., Science, 246:1275 (1989). In this method, mRNA molecules for heavy and light antibody chains are isolated from the immunized animal. The mRNAs are amplified using polymerase chain reaction (PCR) techniques. The nucleic acids are then randomly cloned into lambda phages to generate a library of recombined phage particles. The phages are used to infect an expression host such as E. coli. The E. coli colonies and corresponding phage recombinants can then be screened for those producing the desired Fab fragments. Preferred lambda phage vectors are gt11 and zap 2.

The antibody molecule-containing composition employed in the present invention can take the form of a solution or suspension. The preparation of a composition that contains antibody molecules as active ingredients is well understood in the art. Typically, such compositions are prepared as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which do not interfere with the assay and are compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

An antibody molecule composition can be formulated into a neutralized acceptable salt form. Acceptable salts include the acid addition salts (formed with the free amino groups of the antibody molecule) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

15 G. Diagnostic Assay Methods

The present invention contemplates a method for detecting an EPR-1 molecule, or polypeptide portion thereof. The assay can be for cell surface receptors homologous with EPR-1, including EPR-1 itself. The assay can be specific for EPR-1 itself by a proper selection of antibody specificity. Also, an assay of the invention can be for polypeptide receptors homologous to portions of EPR-1 as well as "free", i.e., unassociated with any particular cell structure, polypeptides homologous to EPR-1, or polypeptide portions thereof. Typically, the assay methods involve detecting EPR-1 exposed on cell surfaces, e.g., CLL cells.

The relative binding affinity of a reagent molecule for its target species is conveniently determined as described herein using the method of flow microfluorimetry (FMF). Thus, cells expressing the target antigen, e.g., EPR-1, are indicated whenever the fluorescence intensity associated with the cells due to binding of the instant fluorescent-

labelled antibodies to cell surface antigens exceeds a predefined threshold level. The labelled antibodies are typically fluorescein isothiocyanate-conjugated (FITC), although other well known fluorescent labels can be used.

The method for detecting an antigenic polypeptide of the present invention preferably comprises formation of an immunoreaction product between the polypeptide and an anti-polypeptide antibody molecule, as disclosed herein. The antigen to be detected can be present in a vascular fluid sample or in a body tissue sample. The immunoreaction product is detected by methods well-known to those skilled in the art. Numerous clinical diagnostic chemistry procedures can be utilized to form the detectible immunocomplexes.

Alternatively, a polypeptide ligand (non-antibody composition) for an instant EPR-1 receptor or polypeptide can be used in the assay method. An exemplary ligand in this aspect of the invention is a labelled factor Xa enzyme. Thus, while exemplary assay methods are described herein, the invention is not so limited.

A preferred assay method of the present invention involves determining the presence of EPR-1 cell surface receptors. Various heterogeneous and homogeneous assay protocols can be employed, either competitive or non-competitive for detecting the presence and preferably amount of cell surface receptors in a body sample, preferably cell-containing sample. The receptor molecules are homologous with human coagulation factors V and VIII or substantial polypeptide portion thereof, so that some care is required in distinguishing an EPR-1 molecule or its polypeptide portion from factors V and VIII. A particularly preferred receptor for assay is EPR-1, as

expressed on CLL cells.

The method comprises admixing a body sample, preferably human, containing cells to be analyzed with a before-described antibody composition that immunoreacts with the receptor molecules. Preferably, the cell sample is washed free of coagulation factors V and VIII prior to the admixing step. The immunoreaction admixture thus formed is maintained under biological assay conditions for a time period sufficient for any cells expressing the antigen to immunoreact with antibodies in the antibody composition to form an antibody-receptor immunocomplex. The immunoreaction product (immunocomplex) is then separated from any unreacted antibodies present in the admixture. The presence, and if desired, the amount of immunoreaction product formed is then determined. The amount of product formed can then be correlated with the amount of receptors expressed by the cells.

Determination of the presence or amount of immunoreaction product formed depends upon the method selected for identifying the product. For instance, a labelled antibody can be used to form a labelled immunocomplex with a receptor molecule of the present invention. The labelled immunocomplex can be quantitated by methods appropriate for detecting the respective label, e.g., fluorescent, radioactive, biotin labels and the like as discussed hereinbelow. Alternatively, an unlabelled antibody can be used to form an unlabelled immunocomplex, which is subsequently detected by immunoreacting a labelled antibody recognizing the unlabelled antibody with the unlabelled immunocomplex. The immunocomplex thereby becomes labelled and can be detected as described above.

Biological conditions used in the instant assays are those that maintain the biological activity of the antibody, EPR-1 cell surface molecule and polypeptide molecules of this invention. Those conditions include
5 a temperature range of about 4°C to about 45°C, preferably about 37°C, at a pH value range of about 5 to about 9, preferably about 7, and an ionic strength varying from that of distilled water to that of about one molar sodium chloride, preferably about that of
10 physiological saline. Methods for optimizing such conditions are well known in the art.

In a preferred embodiment, a body sample to be analyzed is withdrawn from a patient and apportioned into aliquots. At least one aliquot is used for the
15 determination of antigen expression using an antibody composition of the present invention. If desired, a second aliquot can be used for determining reactivity of a control antibody with the sample. The analysis can be performed concurrently but is usually performed
20 sequentially.

In a further aspect of the invention, data obtained in the instant assays are recorded via a tangible medium, e.g., computer storage or hard copy versions. The data can be automatically input and
25 stored by standard analog/digital (A/D) instrumentation that is commercially available. Also, the data can be recalled and reported or displayed as desired for best presenting the instant correlations of data. Accordingly, instrumentation and software
30 suitable for use with the present methods are contemplated as within the scope of the present invention.

The antibody compositions and methods of the invention afford a method of monitoring treatment of
35 patients afflicted with chronic lymphocytic leukemia

(CLL), and other diseases in which expression of receptors homologous to factors V and VIII is correlated with the disease state. For instance, it is found that the frequency of cells expressing an
5 EPR-1 marker is inversely related to the response to treatment of patients suffering from CLL. Additionally, patients afflicted with hairy cell leukemia (HCL) of the EPR-1⁺ type express markers detected by an instant antibody composition, thereby
10 permitting monitoring of treatment.

Accordingly, a method of monitoring a patient's response to treatment is contemplated in which a marker for the disease is detected. The method comprises admixing a body sample containing cells to
15 be assayed for EPR-1 marker with an instant antibody composition according to an assay method described above. The admixture is maintained for a time period sufficient to form an immunoreaction product under predefined reaction conditions. The amount of
20 immunoreaction product formed is correlated to an initial disease state. These steps are repeated at a later time during the treatment regimen thereby permitting determination of the patient's response to treatment, with a decrease in the number of EPR-1
25 molecules expressed on cell surfaces indicating an improvement in the disease state.

H. Diagnostic Systems

Also contemplated within the instant invention is
30 a diagnostic system for performing the described assays. A diagnostic system in kit form of the present invention includes, in an amount sufficient for at least one assay, a composition containing antibody molecules or fragments thereof of the present
35 invention, as a separately packaged reagent, and

preferably with a label able to indicate the presence of an immunoreaction product. Instructions for use of the packaged reagent are also typically included.

5 "Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the
10 like. In one embodiment, a diagnostic system is contemplated for assaying for the presence of EPR-1 receptors expressed on cells in a cell-containing sample.

A preferred kit is provided as an enclosure
15 (package) that comprises a container for anti-EPR-1 antibodies that immunoreact with receptor molecules on cells in the cell sample. Typically, the kit also contains a labelled antibody probe that immunoreacts with the immunocomplex of the anti-EPR-1 antibody and
20 the EPR-1 receptor.

The label can be any of those commonly available, e.g., fluorescein, phycoerythrin, rhodamine, ^{125}I , and the like. Other exemplary labels include ^{111}In , ^{99}Tc , ^{67}Ga , and ^{132}I and nonradioactive labels such as biotin
25 and enzyme-linked antibodies. Any label or indicating means that can be linked to or incorporated in an antibody molecule is contemplated as part of an antibody or monoclonal antibody composition of the present invention. A contemplated label can also be
30 used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with
35 otherwise novel methods and/or systems.

The linking of labels to polypeptides and proteins is well known. For instance, antibody molecules produced by a hybridoma can be labelled by metabolic incorporation of radioisotope-containing amino acids provided as a component in the culture medium. See, for example, Galfre et al., Meth. Enzymol., 73:3-46 (1981). The techniques of protein conjugation or coupling through activated functional groups are particularly applicable. See, for example, Aurameas, et al., Scand. J. Immunol., Vol. 8, Suppl. 7:7-23 (1978), Rodwell et al., Biotech., 3:889-894 (1984), and U.S. Pat. No. 4,493,795.

An instant diagnostic system can also include a specific binding agent. A "specific binding agent" is a chemical species capable of selectively binding a reagent species of the present invention but is not itself an antibody molecule of the present invention. Exemplary specific binding agents are antibody molecules, complement proteins or fragments thereof, protein A and the like that react with an antibody molecule of this invention when the antibody is present as part of the immunocomplex described above.

In preferred embodiments the specific binding agent is labelled. However, when the diagnostic system includes a specific binding agent that is not labelled, the agent is typically used as an amplifying means or reagent. In these embodiments, a labelled specific binding agent is capable of specifically binding the amplifying means when the amplifying means is bound to a complex containing one of the instant reagents.

For example, a diagnostic kit of the present invention can be used in an "ELISA" format to detect the presence or quantity of an EPR-1 polypeptide in a body sample or body fluid sample such as serum, plasma

or urine or a detergent lysate of cells, e.g., a 10mM CHAPS lysate. "ELISA" refers to an enzyme-linked immunosorbent assay that employs an antibody or antigen bound to a solid phase and an enzyme-antigen or enzyme-antibody conjugate to detect and quantify the amount of antibody or antigen present in a sample. A description of the ELISA technique is found in Chapter 22 of the 4th Edition of Basic and Clinical Immunology by D.P. Sites et al., published by Lange Medical Publications of Los Altos, CA in 1982 and in U.S. Patents No. 3,654,090; No. 3,850,752; and No. 4,016,043, which patents are incorporated herein by reference.

In preferred embodiments, the antibody or antigen reagent component can be affixed to a solid matrix to form a solid support that is separately packaged in the subject diagnostic systems. The reagent is typically affixed to the solid matrix by adsorption from an aqueous medium, although other modes of affixation well known to those skilled in the art can be used. For example, an instant anti-EPR-1 antibody can be affixed to a surface and used to assay a solution containing EPR-1 molecules or cells expressing EPR-1 receptors. Alternatively, EPR-1, polypeptide fragments thereof, and whole or partially lysed cells expressing EPR-1 can be affixed to the surface and used to screen a solution for antibody compositions that immunoreact with the affixed species.

Useful solid matrix materials in this regard include the derivatized cross-linked dextran available under the trademark SEPHADEX from Pharmacia Fine Chemicals (Piscataway, NJ), agarose in its derivatized and/or cross-linked form, polystyrene beads about 1 micron to about 5 millimeters in diameter available

from Abbott Laboratories of North Chicago, IL, polyvinyl chloride, polystyrene, cross-linked polyacrylamide, nitrocellulose- or nylon-based webs such as sheets, strips or paddles, tubes, plates, the wells of a microtiter plate such as those made from polystyrene or polyvinylchloride, and the like.

The reagent species, labelled specific binding agent or amplifying reagent of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry powder, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. Usually, the reagents are packaged under an inert atmosphere. A solid support such as the before-described microtiter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

The diagnostic system is contained in a conventional package. Such packages include glass and plastic (e.g., polyethylene, polypropylene and polycarbonate) bottles, vials, plastic and plastic-foil laminated envelopes and the like.

25 Examples

The following examples illustrate but do not limit the invention.

1. Cells and Cell Culture. PMN (polymorphonuclear leucocytes) were isolated by dextran sedimentation from acid-citrate dextrose anticoagulated blood. PBMC (peripheral blood mononuclear cells) were separated after platelet-rich plasma was removed by low speed differential centrifugation over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) (density = 1.077 g/ml) at 400 x g

for 18 minutes at 22°C. PBMC were extensively washed in 5 mM EDTA-PBS, pH 7.2, and incubated twice in autologous serum containing 5 mM EDTA for 30 minutes at 37°C to prevent platelet-monocyte rosetting.

5 Monocytes were isolated from PBMC by adherence to plastic petri dishes precoated with autologous serum for 1 hour at 37°C. Cells were suspended at 1 to 1.5×10^7 /ml in detectable endotoxin-free RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) and 10% heat
10 inactivated FCS (fetal calf serum; Gemini Bioproducts Inc., Calabasas, CA), 2 mM L-glutamine (Irvine), 25 mM HEPES (Calbiochem Boehringer Diagnostic, La Jolla, CA), 100 µg/ml gentamicin (Geramycin, Schering Corp., Kenilworth, NJ).

15 The monocytic cell line THP-1 (ATCC) was maintained in continuous culture in the above media further supplemented with 10 µM 2-ME (Eastman Kodak, Rochester, NY). The transformed human leukemic-
20 lymphoma T cell lines HuT 78, MOLT 4, CCRF-CEM, CCRF-HSB-2, and the human B lymphoma cell lines Raji and Daudi (ATCC) were maintained in culture as recommended. The human leukemia-lymphoma T cell lines
25 Jurkat, MLT, PEER, and MOLT 13 were the generous gift of Dr. D. P. Dialynas, Research Institute of Scripps Clinic, La Jolla, CA.

For mixed lymphocyte response, 20×10^6 freshly isolated PBMC were cultivated in T-25 vented flasks (Costar Corp., Cambridge, MA) in the presence of 20×10^6 irradiated (10,000 rad) Raji or Daudi cells.
30 Cultures were maintained in RPMI 1640, 10% FCS, 25 mM HEPES, 10 µM 2-ME (mixed lymphocyte culture (MLC) medium) in a 5% CO₂ humidified incubator for 7 days at 37°C. Responder cells were harvested, isolated by
35 centrifugation on Ficoll-Hypaque at $400 \times g$ for 18 minutes at 22°C, washed in complete MLC medium, then

cultured in 24-well plates (Costar) at 2×10^5 /well in the presence of 2×10^6 irradiated Daudi or Raji cells.

For long term culture of alloreactive cells stimulated by irradiated Daudi or Raji, responder T cells were transferred every 6 days according to the protocol described above and recultured in MLC medium containing 10% T cell growth factor (Cellular Product Inc., Buffalo, NY). In some studies, suspensions of freshly isolated PBMC at 1×10^6 /ml were treated with $1 \mu\text{g/ml}$ Con A (Calbiochem) or $1 \mu\text{g/ml}$ PHA (Phytohemagglutinin; Calbiochem) for 7 days in 5% CO_2 at 37°C . Aliquots of cells from these cultures were harvested after various time intervals, washed, and analyzed by FMF (flow microfluorometry).

2. mAb. The experimental procedures for purification and characterization of human V have been reported previously in Altieri, D. et al., J. Biol. Chem., 264:2969 (1989). Briefly, BALB/c mice were immunized intraperitoneally with 50 μg of V in CFA (complete Freund's adjuvant; Calbiochem) and hybridomas generated as described previously [Altieri, D. et al. J. Biol. Chem., 264:2969 (1989)]. Screening strategy for antibody selection was to analyze by FMF the reactivity of hybridoma culture fluids with THP-1 cells [Altieri, D. et al., J. Biol. Chem., 264:2969 (1989)]. Six hybridomas reacting with >98% of THP-1 cells were selected for antibody production in solid phase RIA and immunoblotting using immobilized V, and finally established by two to four times sequential subclonings by limiting dilution. A rabbit polyclonal antiserum raised by multiple immunizations with purified V was also screened by FMF and characterized according to the strategy described above. In

addition, a second panel of mAb elicited by immunization with V, reactive with V by Western blot but nonreactive with THP-1 cells by FMF was selected and established.

5 Purified Ig fractions of mAb 7G12 (IgG2a), 9D4 (IgG1), and 12H1 (IgM) (ATCC Accession No. HB 10637) were prepared by chromatography on Affi-Gel MAPS II or hydroxylapatite columns (Bio-Rad, Richmond, CA). Purified Ig fractions of anti-V rabbit polyclonal antiserum B78.9 were prepared by ammonium sulfate
10 fractionation and chromatography on DEAE Sephadex. Immunopurified B78.9 antibodies were isolated from purified factor V immobilized on Affigel 15 (Biorad, Richmond, CA) according to the manufacturer's
15 directions.

Anti-CD16 mAb were Leu 11b (Becton Dickinson, Mountain View, CA) B73.1 and 3G8, the kind gift of Dr. G. Trinchieri, the Wistar Institute, Philadelphia PA. Anti-CD56 mAb NKH-1 (Leu 19) was purchased from
20 Coulter Immunology, Hialeah, FL. Anti-CD11b and anti-CD18 mAbs were OKM1 and 60.3, respectively. mAbs to CD57 (HNK-1), CD3 (OKT3), CD4 (OKT4), CD8 (OKT8), CD2 (OKT11), HLA class I (W6/32) were acquired from ATCC. Anti- α/β T cell receptor (TCR) mAb WT31 was purchased
25 from Becton Dickinson, anti- δ TCR mAb δ 1 was kindly provided by Dr. M.B. Brenner, Harvard Medical School, Boston, MA.

3. Binding Reactions. The interaction of
30 various mAb with different cell types was evaluated by FMF. Briefly, 1×10^6 cells were incubated in V-bottomed microtiter plates (Costar) with saturating concentrations of each mAb for 30 minutes at 4°C. After washes in MLC media, 1/20 dilution aliquots of
35 fluorescein-conjugated goat (F(ab')₂ anti-mouse IgG +

IgM (Tago Inc., Burlingame, CA) were added for an additional 30 minutes at 4°C. Cells were washed and immediately analyzed on a Becton Dickinson IV/40 FACS.

Simultaneous two-color FMP analyses were performed as described previously [Altieri, D. et al., J. Biol. Chem., 264:2969 (1989)] using mAb 7G12 or 9D4 previously conjugated with biotin (N-hydroxysuccinimido-biotin, Sigma) and revealed by 1/20 dilutions of phycoerythrin-conjugated streptavidin reagent (Tago).

To confirm the accuracy of the two-color FMP analysis performed on the various cell populations, two additional sets of studies were also carried out. First, to avoid possible cross-reaction of the second FITC-conjugated anti-mouse reagent with the biotinylated mAb, these studies were repeated by using biotin-conjugated aliquots of the rabbit polyclonal antibody B78.9 in association with the various anti-T cell or anti-NK (natural killer) cell-related markers mAb.

In a further series of studies, directly FITC-conjugated mAb 7G12 or 9D4 (Chromaprobe, Inc., Redwood City, CA) were also used in combination with biotin-conjugated mAb) OKT3, OKT4, OKT8. For cell sorting studies, HuT 78 cells ($1.5 \times 10^7/\text{ml}$) were incubated with the anti-V polyclonal antiserum B78.9 followed by fluorescein-conjugated goat anti-rabbit IgG (Tago). B78.9⁺ HuT 78 cells (HuT 78*, 34% of the unfractionated population) were isolated on a Becton Dickinson Facstar under negative pressure with a sweep rate of 2000 cells/s, washed in complete MLC medium, and cloned by limiting dilution in 96-well round-bottomed plates (Costar) at 0.3, 1, 3 cells/well in HuT 78 conditioned medium supplemented with 20% FCS. After 3 weeks, proliferating cells of single cell

clonal origin on the basis of Poisson distribution were subcloned, established, and further phenotypically characterized by FMF.

The procedures for the isolation, characterization, and ^{125}I -labelling of factor Xa were as described previously by Altieri, D. et al., J. Biol. Chem., 264:2969 (1989). The interaction of ^{125}I -Xa with HuT 78* cells was analyzed by incubating increasing concentrations of ^{125}I -Xa (0.45 to 36 nM) with cell suspension at 1.5 to $2 \times 10^7/\text{ml}$ in the presence of 2.5 mM CaCl_2 for 20 minutes at room temperature. At the end of the incubation, the reaction was terminated by centrifugation of aliquots of the cell suspension at $12,000 \times g$ for 2 minutes through a mixture of silicone oil to separate free from cell-associated radioactivity. Nonspecific binding was quantified in the presence of 50-fold molar excess of unlabelled factor Xa added at the start of the incubation reaction, and was subtracted from the total to yield net specific binding. In some studies, aliquots of HuT 78* cells were preincubated with 50 $\mu\text{g}/\text{ml}$ of mAb 9D4 for 30 minutes at room temperature before the addition of serial concentrations of ^{125}I -Xa.

4. Cell Surface Labelling and Immunoprecipitation. Suspensions of PMN at $1 \times 10^8/\text{ml}$, were surface iodinated with 5 mCi ^{125}I -Na by the Iodogen method [Fraker, P. J., et al., Biochem. Biophys. Res. Commun., 80:849 (1978)]. After extensive washes in HEPES saline buffer pH 7.35 cells were lysed in buffer containing 0.5% Triton X-100 or 10mM CHAPS, 0.05 M Tris HCl, 0.15 M NaCl, 1 mM benzamidine, 0.1 mM (PPACK = D-Phe-Pro-Arg chloromethylketone; Calbiochem), 25 $\mu\text{g}/\text{ml}$ leupeptin, 1mM PMSF (phenylmethyl sulfonyl

fluoride; Calbiochem), pH 8.3 (lysis buffer), for 30 minutes at 4°C. The iodinated lysate was cleared of nuclei and other cellular debris by centrifugation at 14,000 x g for 30 minutes at 4°C, and extensively

5 preabsorbed with aliquots of goat anti-mouse IgG + IgM conjugated with sepharose CL4B (Calbiochem). Aliquots of the ¹²⁵I-labelled PMN lysate were separately incubated with mAb 12H1 or 60.3 for 14 hours at 4°C under agitation. The immune complexes were
10 precipitated by the addition of goat anti-mouse IgG + IgM conjugated with sepharose CL4B for an additional 6 hours at 4°C, extensively washed in the above lysis buffer, and finally resuspended in 2% SDS sample buffer, pH 6.8, containing 50 mM 2-dithiothreitol as a
15 reducing agent. The samples were immediately boiled for 5 minutes, clarified by centrifugation at 14,000 x g for 5 minutes and finally electrophoresed on 7.5% SDS polyacrylamide slab gels in 0.1% SDS. Gels were stained in Coomassie blue R 250, destained in 5%
20 acetic acid, dried and exposed for autoradiography at -70°C by using Kodak X-Omat AR X-Ray film and intensifying screens (Cronex E.I., DuPont de Nemours, Wilmington, DE).

25 5. Isolation of EPR-1 Molecule. The isolation to homogeneity of the EPR-1 molecule required the identification and/or establishment of cell types that constitutively express high levels of this surface antigen. These studies were conducted primarily on
30 peripheral blood polymorphonuclear leukocytes (PMN) and on a specifically selected T cell clonal derivative from the parental T cell line MOLT13 #3 [Altieri, et al., J Immun. 145:246 (1990)].

35 The subline MOLT13 #3 was established by two sequential cycles of fluorescence sorting of the

parental line MOLT 13 using the anti-EPR-1 mAb 12H1. Only MOLT 13 cells expressing the highest levels of reactivity with mAb 12H1 by fluorescence analysis were isolated, cloned by limiting dilutions at 1 or 3 cells/well, grown to confluency, and finally re-screened again by flow cytometry for reactivity with mAb 12H1 as well as with a panel of mAbs directed against various T cell-related markers. The subline MOLT13 #3, established as described above, expressed 7-10 fold higher levels of EPR-1 as compared with the parental line.

MOLT13 #3 cells (ATCC Accession Number CRL 10638) were grown continuously in suspension at 37°C in T150 tissue culture flasks, 5% CO₂. Cells were harvested, washed twice in ice cold phosphate buffered saline supplemented with 5 mM EDTA, pH 7.2, and lysed in lysis buffer for 1 hour at 4°C in continuous agitation.

The composition of the lysis buffer was found to be critical in obtaining satisfactory recovery of the isolated EPR-1 molecule. The detergent used was 0.3% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Calbiochem) in the presence of 1mM CaCl₂ and a cocktail of protease inhibitors, including PMSF (1 mM), p-APMSF (amidinophenylmethyl sulfonyl fluoride; Calbiochem) (1 mM), PPACK (0.25 mg/ml), soybean trypsin inhibitor (0.1 mg/ml), benzamidine (1 mM), leupeptin (0.25 mg/ml), aprotinin (10,000 U/ml). The ratio between volume of lysis buffer and cell number subjected to lysis depends on the cell type selected for the analysis. For MOLT13 #3 cells, approximately 120 ml of lysis buffer were required to effectively solubilize 10⁹ cells. Under the same conditions, 4x10⁹ PMN was effectively solubilized using 200 ml of EPR-1 lysis buffer. The solubilized

cell extract was then cleared of nuclei and other insoluble material by centrifugation at 6,000 rpm for 30 minutes at 4°C, and stored at -70°C until ready to use.

5 The immunoaffinity isolation of the EPR-1 molecule from the detergent-solubilized cell extracts prepared as described above, was based on the use of the anti-EPR-1 mAb 12H1 (IgM isotype). Approximately 4×10^9 cells solubilized as described were incubated
10 with mAb 12H1 for 16 hours at 4°C in agitation. The ratio of mAb 12H1 used was 1 ml of ascites/100 ml of EPR-1 lysate.

 At the end of the incubation, the EPR-1 molecule bound to mAb 12H1 was isolated by the addition of 2 ml
15 aliquots (5 mg) of goat anti-mouse IgM covalently conjugated to Sepharose CL4B (solid phase immuno-
absorbent) for an additional 6 hours at 4°C in agitation. At the end of that time period, the immunoprecipitate was washed five times in EPR-1 lysis
20 buffer by centrifugation at 3,000 rpm for 20 minutes at 4°C.

 After the washes, the pellets from various tubes were pooled, resuspended in 0.4 ml of nonreduced SDS sample buffer, pH 6.8, boiled for 5 minutes to
25 separate the antigen from the antibody complex, and finally separated by electrophoresis on a 7.5% preparative SDS polyacrylamide gel, applying 25
mAmps/gel constant current. After electrophoretic migration, the gels were fixed in methanol, stained in
30 0.08% Coomassie blue, and destained overnight in 10% methanol and 5% acetic acid.

 This isolation procedure allowed visualization of a prominent 78 kDa band stained by Coomassie blue and a second specific component migrating with a 66-68 kDa
35 molecular size and frequently appearing as a doublet

of 66/68 kDa. The 66/68 kDa species are believed due to limited proteolysis or underglycosylation. The 78 kDa stained band was excised from the preparative gel using a razor blade, macerated in small fragments with a spatula, and mixed with a digestion buffer containing 20% glycerol, 0.1% bromophenol blue, 125 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, pH 6.9. The macerated bands were loaded in 6 wells on a 15% SDS-polyacrylamide gel and each sample was overlaid with 40 μ l of Endoproteinase G, sequencing grade (v8) at 100 μ g/ml final concentration.

The samples were prerun at 50 mAmps until they entered the separating gel, digested during a 30 minute incubation time, and the generated fragments were finally separated during the remaining electrophoretic run. The gels were quickly soaked in water after the migration, then in CAPS ([3-(cyclohexylamino)-propanesulfonic acid]; Calbiochem) transfer buffer containing 10 mM CAPS and 20% methanol, and finally assembled onto Immobilon membrane in a gel transfer apparatus. Protein transfer was carried out at 450 mAmps for hours at room temperature. The Immobilon membranes were then removed, soaked in water, stained for 30-60 minutes in Coomassie blue 50% methanol, rinsed in water and destained in 10% acetic acid and 40% methanol. This procedure permitted the direct identification of a number of heavily stained bands by internal cleavage of the 78 kDa band EPR-1 molecule, and subsequent electrophoretic separation of the corresponding fragments.

Bands of interest were excised from the stained transfer on the basis of their relative molecular weight and subjected to NH_2 microsequence analysis using an Applied Biosystems Vapor Phase Sequencer with

on-line HPLC available in the Microchemistry core of the Research Institute of Scripps Clinic. The derived peptide sequences from a number of studies conducted as described above on both PMN and MOLT13 #3 T cells were analyzed by the GENALIGN computer program (Intelligenetics, Inc.) on a SUN computer and by the WORDSEARCH programs (Univ. of Wisconsin Genetics Computer Group) on a VAX 750 computer. As indicated in Table 1, all the deduced peptide sequences derived from the EPR-1 molecule showed a significant degree of homology exclusively with the coagulation proteins, factor V and VIII, and with a recently described murine cell-surface molecule denominated MFG E-8 [Stubbs et al., Proc. Natl. Acad. Sci. U.S.A. 87:8417 (1990)], that shares remarkable homologies with these two coagulation proteins and the EPR-1 molecule.

52

TABLE 1

EPR-1 SEQUENCE ALIGNMENTS*

5	RES. #	SEQUENCE	RES. #	SOURCE
EPR-1 SEQUENCE #1				
10	1	TLKG.QTOGAVMI	12	EPR-1 #1
		:.		
	215	RVSGYMTQGASRA	227	MFG E-8
		:. . .		
	2099	IIHGIKTOGARQK	2111	factor VIII (C1)
15		. . : .		
	1972	IITGIQTQGAHY	1984	factor V (C1)
			
	2033	KITAIITQKDSI	2044	factor V (C2) (Preferred)
		: . : . :		
20	2259	KVTGVTTOGVKSL	2271	factor VIII (C2) (Preferred)
		. : . . .		
	376	QVTGIITQGARDF	388	MFG E-8 (Preferred)
		. : . .		
25	1	TLKG.QTOGAVMI	12	EPR-1 #1
EPR-1 SEQUENCE #2				
	1	PXIXQMDLL	9	EPR-1 #2
30		. :		
	204	PWI QVNLL	211	MFG E-8
		.		
	2089	.WI KVDLL	2095	factor VIII (C1)
		.		
35	1963	PWI QVDMQ	1967	factor V (C1)
		: :		
	204	PWI QVNLL	211	MFG E-8
EPR-1 SEQUENCE #3				
40	1	ACKLREELHKX	11	EPR-1 #3
		: .		
	293	GCTLRFELLGC	303	MFG E-8
45		: . .		
	2178	RSTLRMELMGC	2188	factor VIII (C1)
		.		
	2051	RPTLRLELQGC	2061	factor V (C1)
		. . :		
50	1	ACKLREELHKX	11	EPR-1 #3

53

EPR-1 SEQUENCE #4

	1	VDKLAPRDP.LA	11	EPR-1 #4
		. . : :		
5	173	FMGLQRWQPELA	184	MFG E-8
		. . .		
	2059	SGQYGQWAPKLA	2070	factor VIII (C1)
		. . :		
	1926	SEFLGYWEPRLA	1937	factor V (C1)
10		: . . : :		
	1	VDKLAPRDP.LA	11	EPR-1 #4

EPR-1 SEQUENCE #5

15	274	FNPTLEAQ	281	MFG E-8
		. :		
	1	GVPPVVT	7	EPR-1 #5
		: .		
20	2032	FDPPIVARY	2040	factor V (C1)
		: :		
	2159	FNPPIIARY	2167	factor VIII (C1)
		. : : . :		
	2316	LDPPLLTRY	2324	factor VIII (C2) (Preferred)
25		: :		
	1	GVPPVVT	7	EPR-1 #5
		: : :		
	2192	FNPPIIISRF	2200	factor V (C2) (Preferred)
		: . . . : :		
30	434	FEKPFMARY	442	MFG E-8 (Preferred)

EPR-1 SEQUENCE #6

35	262	GNLDNNSLKVN	272	MFG E-8 (Preferred)
		. :		
	1	GNSDAXYVKXV	9	EPR-1 #6
		:		
	2020	GNSDASTIKEN	2028	factor V (C1) (Preferred)
40		. .		
	2147	GNVDSSGIKHN	2158	factor VIII (C1) (Preferred)
		. .		
	2181	GNTNTKGHVKN	2191	factor V (C2)
		. : : .		
45	2304	GNQDSFTPVVN	2314	factor VIII (C2)
		: .		
	422	GNLDNNSHKKN	430	MFG E-8
	1	GNSDAXYVKXV	9	EPR-1 #6
50				

EPR-1 SEQUENCE #7

	353	INAWTAQSNSAKEWLQVD	370	MFG E-8
		:: . . : . : : . .		
5	1	VQKLAEDENNAKKHVEPH	18	EPR-1 #7
		. : : .		
	2109	VNAWQAKANNNKQWLEID	2126	factor V (C2)
		: : : :		
	353	INAWTAQSNSAKEWLQVD	370	MFG E-8
10		: : : :		
	2109	VNAWQAKANNNKQWLEID	2126	factor V (C2)
		. : . . : : :		
	2235	SNAWRPQVNNPKEWLQVD	2251	factor VIII
		: . . : : : : .		
15	1	VQKLAEDENNAKKHVEPH	18	EPR-1 #7

*Numbering nomenclature for human factor V and factor VIII is from NBRF Protein Data Bank and includes the leader peptides in the sequence (29 residues for factor V and 19 residues for factor VIII). Numbering of MFG E-8 is from Stubbs et al. Proc. Nat. Acad. Sci., 87:8417-8421 (1990), but includes the 22 residue leader peptide. The factor V and factor VIII sequence numbering in Stubbs et al. is the NBRF scheme minus 29 for factor V and minus 19 for factor VIII. | = identity; : = conservative substitution; . = semi-conservative substitution. X is a variable amino acid residue. A space, where present in a sequence, is a gap introduced to improve sequence alignments. Note that factor V and factor VIII have two repeated highly homologous C domains and sequences, thus there are duplicate homology matches. The domain matches with C1 or C2 and the preferred homology are indicated when both must be considered.

6. EPR-1 Expression Correlates with Response to CLL Therapy. The reactivity of anti-EPR-1 mAbs with peripheral blood cells isolated from patients with hematopoietic malignancies was explored with flow cytometry. It was found that in 28 out of 37 CLL patients (75%), the number of EPR-1⁺ cells was increased 5-6 fold thereby including most of the circulating population, as compared with normal controls (EPR-1⁺ cells in normal donors: 16.5±3.2%, n-12 versus EPR-1⁺ cells in CLL: 89.1±2.5%, n-28).

The number of EPR-1 molecules expressed on CLL cells also showed a mean increase of 2.5 fold as compared with normal controls (mean fluorescence of EPR-1⁺ normal cells: 85.6±16.1 versus EPR-1⁺ CLL:

5 215.6±50.3). Roughly 98% of PMC cell were positive to this marker. Two-color flow cytometry studies confirmed that in CLL patients both CD5 and EPR-1 were simultaneously co-expressed in the same cell population. Finally, sequential analysis of a group
10 of CLL patients carried out over a 4-month period (starting at day 0) showed that positive biologic response to the therapy was frequently associated with drastic reduction (67-90% reduction) in the number of EPR-1⁺ cells detected. Representative patient data
15 are presented in Table 2, below, and illustrate this trend.

EPR-1 therefore represents a novel cellular marker in CLL and its surface expression inversely correlates with the patient's biologic response to the
20 therapy. The data further emphasize the possible participation of protease-mediated mechanisms in the development and/or establishment of selected hematopoietic malignancies.

25

TABLE 2

EPR-1 Expression on Leukemia Cells*

Patient #	Day	% 12H1	% B78.9
30	1	0	91.5
	35	95.3	11.4
	70	9.2	49.6
35	2	0	46.8
	41	98.7	98.6
	75	5.2	32.8
3	0	98.3	92.6
	36	66.7	83.7

56

5	4	0	25.4	32
		28	46.3	51.8
		56	2.9	1.4
		84	63	41.2
10	5	0	99.7	98.9
		55	21	43.8
15	6	0	94	31.2
		35	9.6	88.9
20	7	0	97	96.8
		34	75.8	87
25	8	0	96.7	88.4
		34	92.6	77.2
		69	82.2	12.2

* 12H1 and B78.9 are monoclonal and polyclonal antibodies, respectively, as described above. The data presented are percentages of cells examined which expressed suprathreshold amounts of EPR-1.

7. PCR Generation of EPR-1 DNA. Using the amino acid residue sequences obtained for EPR-1 presented in Table 1, primer molecules were identified for PCR amplification of nucleic acids coding for the EPR-1 polypeptides, including EPR-1 itself. For instance, oligonucleotides that anneal with the (-) strand of DNA and that code for EPR-1 #1 and #3 amino acid residue sequences are:

#1 5'-AAAGGICAGACICAGGGIGCIGTIATGAT-3';

#3 5'-TGCAAAITTIIGIGAAGAAITICACAAA-3'.

Probes complementary to those identified above can be used to hybridize with a (+) strand of RNA or DNA. Generally, one of the primers listed above should be used with a primer complementary to the other primer listed above so that extension products on both the (+) and (-) strands are generated simultaneously. Also, the primers can be extended to include convenient restriction and cloning sites as

desired. Since a #1 primer can anneal 5' and/or 3' to the #3 primer, usually both sets of primers and primer complements will be used in a PCR protocol. The reaction conditions and cycling protocol for PCR are well known and are described above. In these nucleotide probes, A is adenine, G is guanine, C is cytosine, T is thymine, and I is inosine.

8. Discussion of Examples 1-7.

10 Cellular Distribution of EPR-1. Monoclonal antibodies against V, the circulating plasma protein that binds the Xa serine protease of coagulation cascade Xa were prepared [Nesheim, M., et al., J. Biol. Chem., 254:10952 (1979)]. In a previous study, it was shown
15 that a minor fraction of these mAb (mAb panel I 7G12, 9D4, and 12H1) also reacts with a surface molecule expressed on various myeloid monocytic cell lines [Altieri, D. et al., J. Biol. Chem., 264:2969 (1989)]. Using mAb inhibition studies and receptor-ligand
20 chemical cross-linking, it has been demonstrated that this cell-associated immunoreactive molecule functions as a high affinity ($K_d = 30$ nM, n about 150,000) receptor for Xa [Altieri, D. et al., J. Biol. Chem., 264:2969 (1989)]. In this study, mAb panel I was
25 exploited to characterize the cellular distribution and identity of the putative membrane serine protease receptor (EPR-1).

As illustrated in Figure 1, the reactivity of panel I anti-V mAb is not an eccentric characteristic
30 of transformed in vitro cell lines. mAb 9D4, recognizing a different epitope from the one previously identified by mAb 7G12 [Altieri, D. C., J. Biol. Chem., 264:2969 (1989)], reacted with peripheral blood monocytes and dextran-isolated PMN, although
35 with considerable heterogeneity in the latter

population (Fig. 1).

When suspensions of PBMC were analyzed by FMF, mAb 7G12, 9D4, and 12H1 consistently reacted with a population of cells (5 to 20%) with forward light scatter characteristic of lymphocytes. Simultaneous two-color FMF analyses were performed to further dissect the phenotype of this lymphoid population. For these studies, suspensions of PBMC were preparatively depleted of adherent cells by either adherence to plastic or by nylon wool fractionation to yield populations enriched in PBL (peripheral blood lymphocyte). Approximately 50 percent of the lymphoid subset identified by mAbs 7G12, 9D4, or 12H1 was OKT3⁻ and expressed the NK-associated markers CD16 and CD56, as revealed by the simultaneous binding of mAb Leu 11b, 3G8, B73.1, and NKH-1, respectively. Furthermore, when enriched populations of NK cells (<3%CD3⁺, >85%CD16⁺) prepared from PBMC by nylon wool fractionation, SRBC (sheep red blood cells) rosetting, and negative selection with mAb OKT3, were analyzed by FMF, mAb 7G12 and 9D4 reacted with 68 and 72% of these cells.

The remaining EPR-1⁺ PBL were phenotypically established as CD3⁺ lymphocytes. Table 3, hereinafter, shows a representative study of two-color FMF characterization of this EPR-1⁺ subset. Although double-positive cells coexpressing either CD4 or CD8 were identified, the latter fraction consistently exhibited a higher frequency and a far greater intensity of reaction with EPR-1 marker mAb.

Virtually all EPR-1⁺ T cells also coexpressed CD11b and CD57(Leu 7), as revealed by mAb OKM1 and HNK-1 respectively, and approximately 70 to 80 percent were CD2⁺(OKT11) (Table 3). Although the EPR-1⁺ subset was predominantly WT31⁺, approximately 10% of EPR-1⁺

cells (2% of unfractionated PBL, n = 3) were found to be reactive with anti- δ TCR mAb δ 1. Quantitatively comparable results were also obtained when two-color FMF analyses of PBL were carried out using biotin-conjugated aliquots of the rabbit polyclonal antibody B78.9, or the directly FITC-conjugated mAb 7G12, or 9D4, in combination with the various anti-T cell or anti-NK cell related markers mAb.

TABLE 3

Two-color FMF characterization of EPR-1⁺ subset of T cells^a

mAb	Specificity	Percent Coexpressing PBL	Relative Percent Coexpressing Cells in EPR-1 ⁺ Subset
OKT3	CD3	9.6	73
OKT4	CD4	3.6	27
OKT8	CD8	7.4	56
OKT11	CD2	10.5	79
OKM1	CD11b	10.8	82
HNK-1	CD57	11.5	88
60.3	CD18	10.7	82
WT31	α/β TCR	8.1	61
δ 1	δ TCR	1.4	10
W6/32	Class I MHC	12.0	91

^aTwo-color FMF analysis of adherent cell-depleted PBL was carried out as follows: Suspensions of PBL were depleted of adherent cells and separately stained with aliquots of anti-CD16 mAb Leu 11b, B73.1, or 3G8, or with anti-CD56 mAb NKH-1 (Leu 19) for 30 minutes at 4°C. Cells were washed and incubated with fluorescein-conjugated goat (F(ab')₂ anti-mouse IgG + IgM for additional 30 minutes at 4°C. After extensive washes, cells were equilibrated with 10 μ g/ml of biotinylated mAb 7G12, washed, and incubated with 1/20 dilution of phycoerythrin-streptavidin conjugated reagent. Double-positive cells from a representative study are indicated for the unfractionated PBL population and relative to the EPR-1⁺ subset (13.1%).

EPR-1 is distinct from CD11b/CD18. The

expression of EPR-1 on monocytes, PMN, NK cells, and a fraction of T cells that is also predominantly CD8⁺, appears to mimic the cellular distribution of the leukocyte integrin CD11b/CD18 (Mac-1) [Sanchez Madrid, F., et al., J. Exp. Med., 158:1785 (1983)].

Therefore, additional studies were designed to establish the reciprocal structure and functional properties of CD11b/CD18 and EPR-1. For these studies, suspensions of PMN that express abundant levels of the CD11/CD18 molecules [Sanchez Madrid, F., et al., J. Exp. Med., 158:1785 (1983)] were surface labelled with ¹²⁵I, detergent-solubilized, and subjected to immunoprecipitation using either the anti-CD18 mAb 60.3 or the anti-EPR-1 mAb 12H1.

From ¹²⁵I-labelled PMN lysate, mAb 60.3 immunoprecipitated the polypeptides corresponding to the α subunits of the leukocyte integrins CD11a, CD11b, and CD11c in association with the common β -subunit CD18, in agreement with previous observations [Sanchez Madrid, F., et al., J. Exp. Med., 158:1785 (1983)]. In contrast, under the same conditions, mAb 12H1 immunoprecipitated a major surface component having a molecular mass of about 78 \pm 4 kDa.

Functionally, CD11b/CD18 and EPR-1 have different ligand recognition specificities. Although CD11b/CD18 has been recognized as an oligo-specific receptor for C3bi, fibrinogen, and factor X [Sanchez Madrid, F., et al., J. Exp. Med., 158:1785 (1983); Altieri, D. et al., J. Cell Biol., 107:1893 (1988); Wright, S. D., et al., Proc. Natl. Acad. Sci. USA, 85:7734 (1988); Altieri, D. et al., J. Biol. Chem., 263:7007 (1988)], EPR-1 binds the activated serine protease Xa [Altieri, D. et al. J. Biol. Chem., 264:2969 (1989)].

Anti-CD11b/CD18 mAb do not inhibit EPR-1 receptor function and the reverse also applies for EPR-1 mAb on

CD11b/Cd18 ligand recognition. Similarly, soluble CD11b/CD18 ligands such as fibrinogen [Altieri, D. et al., J. Cell Biol., 107:1893 (1988); Wright, S. D., et al., Proc. Natl. Acad. Sci. USA, 85:7734 (1988)], and factor X [Altieri, D. C., et al., J. Biol. Chem., 263:7007 (1988)], do not compete or inhibit EPR-1 receptor recognition of Xa.

Dynamic Regulated Expression of EPR-1 on PBL.
Additional studies were designed to explore the possibility of a dynamic modulation of EPR-1 expression under conditions of antigen-specific or mitogen-driven T cell activation. Freshly isolated PBMC were set up in unidirectional mixed lymphocyte culture (MLC) against irradiated allogeneic B cells, i.e., Raji (MHC class I and II driven) or Daudi (MHC class II driven). After 7 days culture, responder T cells were harvested, washed, and phenotypically characterized by FMF using mAb 7G12, 9D4, and 12H1.

In another series of studies, PBMC were separately cultivated for 7 days in the presence of 1 μ g/ml of the polyclonal activators (PHA) or Con A then subjected to FMF analysis. As shown in Figure 2, both allogeneic expansion of normal PBMC or lectin activation resulted in a consistent three- to four-fold increase in EPR-1⁺ T cells, as recognized by mAb 12H1.

To exclude the possibility that the observed expansion of 12H1⁺ cells resulted from a selective redistribution of T cell subsets occurring upon activation, Con A-stimulated PBMC were sequentially analyzed by FMF after various time intervals of culture. Con A-mediated quantitative expansion of the EPR-1⁺ subset occurred in cells with forward light scatter characteristic of proliferating, activated blasts. The number of these cells increased

approximately four-fold between day 6 and 7 of culture and when these cells were phenotypically characterized by two-color FMF they were CD3⁺, CD4⁻, CD8⁺, CD2⁺.

Additional studies were carried out to
5 investigate the effects of long term alloreactive stimulation on EPR-1 expression. Unidirectional MLC against irradiated Daudi cells was maintained in continuous culture with weekly transfers in the presence of 10% T-cell growth factor (TCGF). At
10 various time intervals, aliquots of responder T cells were harvested, recovered by centrifugation over Ficoll-Hypaque, and finally analyzed for EPR-1 marker expression by FMF using mAb 12H1 or the polyclonal antiserum B78.9. These data are summarized in Figure
15 3. The number of EPR-1⁺ cells detected by mAb 12H1 increased approximately ninefold during antigen-mediated activation after one month of culture. Similar results were also obtained using the polyclonal antiserum B78.9, which shows a larger
20 reactivity consistent with the greater number of EPR-1 epitopes detected by this reagent.

To distinguish between selective expansion of EPR-1⁺ cells or de novo expression of this marker resulting from polyclonal or antigen stimulation, an
25 additional set of studies was carried out. Suspensions of freshly isolated PBL were preparatively fractionated in EPR-1⁺ and EPR-1⁻ subsets by FMF sorting with mAb 12H1. These resulting populations were then separately cultivated for 10 days with 1
30 µg/ml Con A, 5 µg/ml PHA, or stimulated in mixed lymphocyte response with irradiated Daudi in the presence of 10% TCGF before FMF analysis of EPR-1 expression. The results of these experiments are shown in Table 4, hereinafter. Both polyclonal- or
35 antigen-stimulation of the negatively selected EPR-1⁻

subset was associated with de novo expression of EPR-1 as detected by binding of mAb 12H1.

TABLE 4

5 De novo EPR-1 expression on negatively selected EPR-1⁻ subset activated after short term culture*

10	Stimulation	EPR-1 ⁻ Subset		EPR-1 ⁺ Subset	
		Positive Cells (%)	Fluorescence (U)	Positive Cells (%)	Fluorescence (U)
	---	0.2	5.5	77.5	102.1
	PHA	6.8	78.3	ND	ND
	Con A	47.2	218.5	ND	ND
15	Daudi MLC	45	82	91.8	83.1

20 * Freshly isolated PBL were fractionated in EPR-1⁻ and EPR-1⁺ subsets by FMF using mAb 12H1. The resulting populations were cultivated in the presence of 1 µg/ml Con A, 5 µg/ml PHA or stimulated in allogeneic MHC with Daudi cells for 10 days before FMF analysis with anti-EPR-1 mAb 12H1. U = arbitrary units.

25 EPR-1 Expressed on T Cells is Functionally Active
Protease Receptor. To further substantiate the expression of EPR-1 on discrete lymphoid populations, a number of transformed in vitro T cell lines were screened by FMF using the panel of mAb described above. As shown in Figure 4, of the various T cell
30 lines assayed only a subpopulation of HuT 78 cells was reactive with mAb 7G12. These cells were isolated to >90% purity by fluorescence sorting using the polyclonal antiserum B78.9 to yield the subpopulation HuT 78*, which was then cloned by limiting dilution.
35 Three clones were established, subcloned, phenotypically characterized by FMF as OKT3⁺, OKT4⁺, OKT8⁻, 12H1⁺, B78.9⁺, and one of them was selected for further investigations.

40 When suspensions of HuT 78* were equilibrated with increasing concentrations of ¹²⁵I-Xa in the presence of 2.5 mM CaCl₂, these cells bound the

offered ligand in a specific and concentration-dependent reaction, approaching steady saturation at 30 to 36 nM of added ^{125}I -Xa (Table 5). Quantitatively similar to the results previously obtained with THP-1 cells [Altieri, D. et al., *J. Biol. Chem.*, 264:2969 (1989)], this reaction was regulated by an apparent K_d on the order of 10 to 20 nM, and was saturated when $194,000 \pm 26,000$ molecules of ^{125}I -Xa were specifically associated with the surface of each HuT 78* cell. Finally, preincubation of HuT 78* cells with saturating amounts of mAb 9D4 inhibited specific binding of ^{125}I -Xa to these cells.

TABLE 5

^{125}I -Factor Xa added (nM) *	^{125}I -Xa bound(molecules/cell $\times 10^{-3}$)	
	mAb 9D4	No mAb
1.5	1	6
5	8	26
8	20	50
18	33	140
27	58	170
36	76	200

* ^{125}I -factor Xa binding to HuT 78* cells. HuT 78* cells reacting with the rabbit polyclonal antiserum B/8.9 were isolated to 94.2% purity by fluorescence sorting and cloned by limiting dilution. Three clones were established, phenotypically characterized and one {HuT 78*-3} selected for further investigations. Suspensions of HuT 78*-3 cells at $1 \times 10^7/\text{ml}$ were separately incubated with control antibody or with 50 $\mu\text{g}/\text{ml}$ of the anti-EPR-1 mAb 9D4 for 30 minutes at room temperature, before the addition of increasing concentrations of ^{125}I -factor Xa (0.45 to 36 nM) and 2.5 mM CaCl_2 for additional 20 min at room temperature. The reaction was terminated by centrifugation through mixture of silicone oils and ^{125}I -Xa specific binding to HuT 78* cells was calculated in the presence or absence of anti-EPR-1 mAb 9D4.

Conclusion

The reactivity of a panel of mAb with a cell surface protease receptor expressed on some leukocytes has been characterized. In previous studies, it was shown that a mAb originally raised against the plasma coagulation protein V (7G12) bound in specific and saturable reaction to the monocytic-myeloid cell lines THP-1, U937, and HL-60 [Altieri, D. et al., J. Biol. Chem., 264:2969 (1989)]. Further, by analogy with the known acceptor/cofactor function of the plasma protein Va [Nesheim, M. E., et al., J. Biol. Chem., 254:10952 (1979)], the molecule recognized by mAb 7G12 on these cells appeared to be implicated in a specific receptor function for the serine protease Xa.

A panel of anti-V mAb has now been raised. The hybridomas secreting those mAb were selected by FMF analysis of THP-1 cells, and the mAb were used as probes to search for expression of the V cell surface cross-reacting molecule on peripheral blood cells.

The first conclusion that can be drawn from these studies is that the molecule recognized by these mAb, operatively defined as EPR-1, is not inappropriately expressed only by transformed cell lines in culture. Rather, it has a broad cellular distribution and a remarkable association with cells of myeloid and lymphoid lineage. Although with considerable heterogeneity among the various populations examined, these mAb defining EPR-1 were found to be reactive with peripheral blood monocytes, PMN, and CD3⁻ CD16⁺CD56⁺ NK cells.

Interestingly, a small fraction of circulating T cells was also identified as EPR-1⁺. Phenotypic characterization of this subset by FMF suggested that the expression of EPR-1 does not appear to be segregated into a unique subpopulation defined by

currently known markers of T cells. Although the majority of EPR-1⁺ T cells isolated from various donors was also CD8⁺ or α/β TCR⁺, cells coexpressing CD4 or γ/δ TCR were identified as well. In agreement with this finding, FIMF analysis of various transformed T cell lines in vitro, revealed expression of EPR-1 markers on MOLT 13 cells that were further phenotypically established as CD4⁺ and TCR γ/δ ⁺, respectively, in agreement with previous observations [Lefranc, M. P., et al., Nature, 316:464 (1985); Brenner, M. B., et al., Nature, 325:689 (1987)].

Within the CD8⁺ fraction of normal PBL, EPR-1 expression was consistently associated with coexpression of CD11b(Leu 15) and CD57(Leu 7), as identified by mAbs OKM1 and HNK-1. In earlier studies, this pattern of markers has been associated with suppressor function [Clement, L. T., et al., J. Immunol., 133:2461 (1984); Fox, E. J., et al., J. Exp. Med., 166:404 (1987); Takeuchi, T., et al., Cell. Immunol., 111:398 (1988)] and LAK activity [Dianzani, et al., Eur. J. Immunol., 19:1037 (1989)]. However, at variance with the previously reported poor proliferative response of this T cell subset [Fox, E. J., et al., J. Exp. Med., 166:404 (1987)], EPR-1 expression is observed as strongly increased by both mitogen and antigen stimulation.

This finding appeared to be particularly emphasized in studies using long term cultures of alloreactive-stimulated T cells, where the anti-EPR-1 rabbit polyclonal antibody B78.9 reacted with virtually all responder cells after one month culture. Similarly, de novo EPR-1 expression was also observed after short term polyclonal or antigen stimulation of preparatively sorted EPR-1⁺ populations. Although these data would appear to be compatible with the

hypothesis that EPR-1 is a true T cell activation responsive molecule, further investigations at the single clonal cell level are necessary to conclusively address this possibility. Finally, in agreement with the expression of both CD4 or CD8, no preferential expansion of EPR-1⁺ cells was observed by either class I or class II MHC allogeneic stimulation.

Although the cellular distribution of EPR-1 closely resembles that of the leukocyte integrin CD11b/CD18 [Sanchez Madrid, F., et al., J. Exp. Med., 158:1785 (1983)], structure/function analyses revealed by immunoprecipitation studies and ¹²⁵I-labelled ligand binding assays clearly demonstrate that these are two different molecules implicated in distinct and different receptor recognition functions [Altieri, D. et al., J. Biol. Chem., 264:2969 (1989); Sanchez Madrid, F., et al., J. Exp. Med., 158:1785 (1983); Altieri, D. C., et al., J. Cell Biol., 107:1893 (1988); Wright, S. D., et al., Proc. Natl. Acad. Sci. USA, 85:7734 (1988); Altieri, D. C., et al., J. Biol. Chem., 263:7007 (1988)].

This study has not been designed to address the reciprocal relationship between cellular EPR-1 and the plasma protein V, that originally served as an immunogen to raise the anti-EPR-1 mAb used. However, it is important to note that the anti-EPR-1 mAb panel described (panel I), constitutes only a minor fraction of the anti-V hybridomas elicited by immunization with factor V. In fact, a second panel of anti-V mAb raised and established under identical protocols and selected for production of mAb immunoreactive with factor V did not exhibit cross-reactivity with THP-1 cells. Furthermore, the size (78±4 kDa) and structural organization of EPR-1 resolved in immunoprecipitation studies exhibits remarkable size

similarity to the light chain of the plasma protein factor Va [Nesheim, M. E., et al., J. Biol. Chem., 254:10952 (1979)]. On the basis of these considerations, it is thought that EPR-1 represents a cell surface molecule homologous to the plasma coagulation protein V maintaining some conserved immunoreactive epitopes functionally associated with ligand recognition.

Whether the expression of EPR-1 on various leukocyte populations implies its involvement in specific immune effector functions is presently not known. However, the observation that NK cells and CD8⁺ T cells express a high affinity serine protease receptor is provocative in view of the identification of a family of closely related serine proteases (granzymes) contained in the granules of human and mouse NK and CTL clones [Masson, D., et al., Cell, 49:679 (1987)]. These enzymes share significant homology with a number of serine proteases, particularly with the coagulation proteases factor IXa, Xa, and plasmin [Jenne, D., et al., Proc. Natl. Acad. Sci. USA, 85:4814 (1988); Gershenfeld, H. K., et al., Science, 232:854 (1986); Jenne, D., et al., J. Immunol., 140:318 (1988); Lobe, C. G., et al., Science, 232:858 (1986); Gershenfeld, H. K., et al., Proc. Natl. Acad. Sci. USA, 85:1184 (1988)]. It is also noteworthy that dynamic modulation of gene expression and secretion of the granzymes is increased by the same stimuli that are associated with increased EPR-1 expression in vitro, i.e., long term response to antigen and IL-2 [Manyak, C. L., et al., J. Immunol., 142:3707 (1989); Masson, D., et al., EMBO J., 4:2533 (1985)]. Although the role of the cellular granzymes in NK or CTL killing remains to be elucidated [Dennert, G., et al., Proc. Natl. Acad. Sci. USA,

84:5004 (1987)], a putative role for serine proteases in the lytic process has been suggested by experiments using serine proteases inhibitors [Redelman, D., et al., J. Immunol., 124:870 (1980); Chang, T. W., et al., J. Immunol., 124:1028 (1980); Suffys, P., et al., Eur. J. Biochem., 178:257 (1988); Scuderi, P., J. Immunol., 143:168 (1989)].

By analogy with the general concept of receptor-mediated amplification of proteolytic activities [Miles, L. A., et al., Fibrinolysis, 2:61 (1988); Morrissey, et al., Cell, 50:129 (1987); Nesheim, M. E., et al., J. Biol. Chem., 254:10952 (1979)], it is thought that locally released granzymes might interact with a membrane component on the effector cell to deliver optimal catalytic efficiency, protected from neutralization by circulating protease inhibitors. In this context, EPR-1 would embody the requirements for a surface receptor expressed by immune effector cells, displaying ligand recognition for a prototypical and highly conserved serine protease such as factor Xa, and dynamically up-regulated by antigenic stimulation.

In conclusion, by using an uncommon strategy for mAb selection, a new leukocyte marker, a serine protease receptor, and an apparent cell-surface homologue of the plasma coagulation protein V have been identified. Because of its remarkable distribution on immune effector cells, the name "EPR-1" is proposed to tentatively identify this molecule. Although the role of EPR-1 in the mechanism of cell-mediated formation of fibrin is highlighted by its recognition for Xa [Nesheim, M. E., et al., J. Biol. Chem., 254:10952 (1979)], it is thought that the wide spectrum of biologic activities mediated by serine proteases implicates the involvement of EPR-1 in additional ligand(s) recognition and cell-mediated

functions.

While the present invention is described in some detail by way of illustration and example for purposes of clarity, certain obvious modifications can be
5 practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A purified protein having a molecular weight of about 78 kD that includes the following amino acid residue sequences:

- 5 Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
 Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
 Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
 Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
 Gly-Val-Pro-Pro-Val-Val-Thr;
10 Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
 Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
 Lys-His-Val-Glu-Pro-His-Ala-Thr,

wherein Xaa is an unspecified amino acid residue.

2. The purified protein according to claim 1
15 that further immunoreacts with monoclonal antibodies
 secreted by hybridoma 12H1 having ATCC Accession No.
 HB 10637.

3. An isolated DNA segment coding for a protein
 having a molecular weight of about 78 kDa and that
20 includes the following amino acid residue sequences:

- Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
 Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
 Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
 Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
25 Gly-Val-Pro-Pro-Val-Val-Thr;
 Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
 Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
 Lys-His-Val-Glu-Pro-His-Ala-Thr,

wherein Xaa is an unspecified amino acid residue.

- 30 4. A self-replicating DNA molecule that includes
 a DNA segment coding for a protein having a molecular
 weight of about 78 kDa and that includes the following
 amino acid residue sequence:

- Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
35 Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;

Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
Gly-Val-Pro-Pro-Val-Val-Thr;
Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
Lys-His-Val-Glu-Pro-His-Ala-Thr,

wherein Xaa is an unspecified amino acid residue.

5. A polypeptide comprising up to about 600 amino acid residues having an amino acid residue sequence selected from the group consisting of:

Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
Gly-Val-Pro-Pro-Val-Val-Thr;
Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
Lys-His-Val-Glu-Pro-His-Ala-Thr,

wherein Xaa is an unspecified amino acid residue.

6. An isolated DNA segment coding for a polypeptide comprising up to about 600 amino acid residues having an amino acid residue sequence selected from the group consisting of:

Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
Gly-Val-Pro-Pro-Val-Val-Thr;
Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
Lys-His-Val-Glu-Pro-His-Ala-Thr,

wherein Xaa is an unspecified amino acid residue.

7. A self-replicating DNA molecule that includes a DNA segment coding for a polypeptide comprising up to about 600 amino acid residues having an amino acid

residue sequence selected from the group consisting of:

Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
5 Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
Gly-Val-Pro-Pro-Val-Val-Thr;
Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
10 Lys-His-Val-Glu-Pro-His-Ala-Thr,

wherein Xaa is an unspecified amino acid residue.

8. A polypeptide that immunoreacts with an antibody produced by the 12H1 hybridoma having ATCC Accession No. HB 10637 and which polypeptide includes
15 an amino acid residue sequence selected from the group consisting of:

Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
20 Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
Gly-Val-Pro-Pro-Val-Val-Thr;
Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
Lys-His-Val-Glu-Pro-His-Ala-Thr,

25 wherein Xaa is an unspecified amino acid residue.

9. The polypeptide of claim 8, wherein the polypeptide binds human factor Xa.

10. The polypeptide of claim 8, wherein the polypeptide has a molecular weight of 78 ± 4 kDa.

30 11. The polypeptide of claim 8, wherein the polypeptide is isolated from a cell line selected from the group consisting of THP-1, neutrophils, NK cells, and MOLT 13 #3 having ATCC Accession No. CRL 10638.

12. An antibody composition having an antibody
35 combining site that immunoreacts with a protein

isolated from the MOLT13 #3 cell line having ATCC Accession No. CRL 10638 which protein has a molecular weight of about 78 kDa and includes the following amino acid residue sequences:

5 Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
 Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
 Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
 Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
 Gly-Val-Pro-Pro-Val-Val-Thr;
10 Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
 Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
 Lys-His-Val-Glu-Pro-His-Ala-Thr,

where Xaa is an unspecified amino acid residue.

15 13. The antibody composition of claim 12, which
is produced by the 12H1 hybridoma having ATCC
Accession No. HB 10637.

14. A method of assaying for the presence of a cell surface receptor in a body sample that comprises the steps of:

20 (a) admixing a body sample containing cells to
be assayed for said cell surface receptor and
substantially free of factors V and VIII with an
antibody composition that includes an antibody
combining site which immunoreacts with a protein that
25 includes the following amino acid residue sequences:

 Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
 Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
 Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
 Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
30 Gly-Val-Pro-Pro-Val-Val-Thr;
 Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
 Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
 Lys-His-Val-Glu-Pro-His-Ala-Thr,

wherein Xaa is an unspecified amino acid residue;

35 (b) maintaining said admixture for a time period

sufficient to form an immunoreaction product; and

(c) determining the presence of said product and thereby the presence of the receptor in said body sample.

5 15. The method of claim 14 wherein a portion of said body sample is affixed to a solid support, said immunoreaction admixture comprises a solid phase and a liquid phase, and said immunoreaction product forms in the solid phase.

10 16. The method of claim 14 wherein the protein is isolated from the MOLT 13 #3 cell line having ATCC Accession No. CRL 10638.

15 17. The method of claim 14 wherein the antibody composition comprises antibodies produced by the 12H1 hybridoma having ATCC Accession No. HB 10637.

18. A method of monitoring response of a patient afflicted with a disease condition correlated to EPR-1-expressing tumor cells to a treatment protocol comprising:

20 (a) admixing a body sample from the patient with an antibody composition that includes an antibody combining site which immunoreacts with a protein that includes the following amino acid residue sequences:

25 Thr-Leu-Lys-Gly-Gln-Thr-Gln-Gly-Ala-Val-Met-Ile;
Pro-Xaa-Ile-Xaa-Gln-Met-Asp-Leu-Leu;
Ala-Cys-Lys-Leu-Arg-Glu-Glu-Leu-His-Lys;
Val-Asp-Lys-Leu-Ala-Pro-Arg-Asp-Pro-Leu-Ala;
Gly-Val-Pro-Pro-Val-Val-Thr;
Gly-Asn-Ser-Asp-Ala-Xaa-Tyr-Val-Lys-Xaa-Val; and
30 Val-Gln-Lys-Leu-Ala-Glu-Asp-Glu-Asn-Asn-Ala-Lys-
Lys-His-Val-Glu-Pro-His-Ala-Thr,

wherein Xaa is an unspecified amino acid residue;

(b) maintaining said admixture for a time period sufficient to form a first immunoreaction product;

35 (c) determining a first amount of immunoreaction

product formed;

(d) repeating steps (a)-(b) on a second body sample from the patient after a period of treatment;

5 (e) determining a second amount of immunoreaction product formed; and

(f) determining the difference between said first and second amounts of immunoreaction products formed and thereby the response of the patient to the treatment protocol.

10 19. The method of claim 18, wherein the antibody composition comprises antibodies produced by the 12H1 hybridoma having ATCC Accession No. HB 10637.

15 20. The method of claim 18, wherein the antibody composition immunoreacts with a protein isolated from a cell line selected from the group consisting of THP-1, monocytes, neutrophils, NK cells, and MOLT 13 #3 having ATCC Accession Number CRL 10638.

1/5

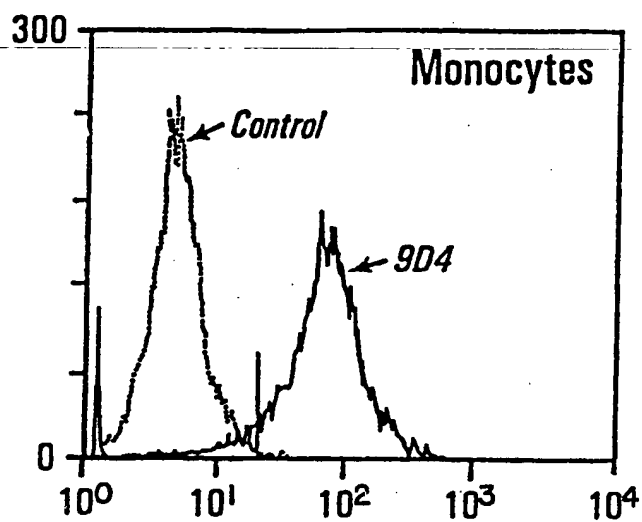


FIG. 1A

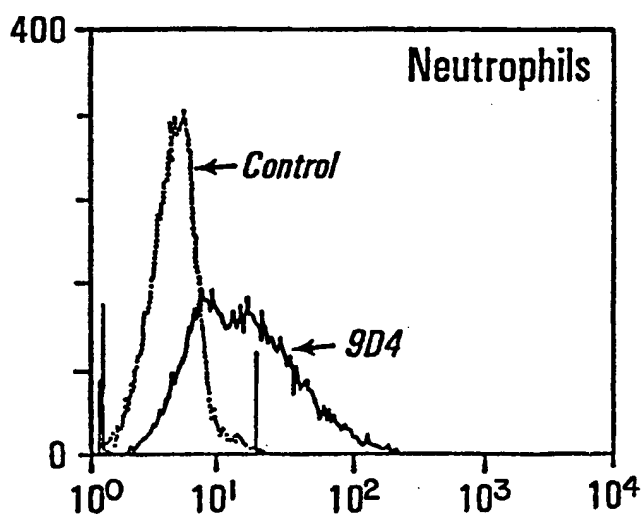


FIG. 1B

2 / 5

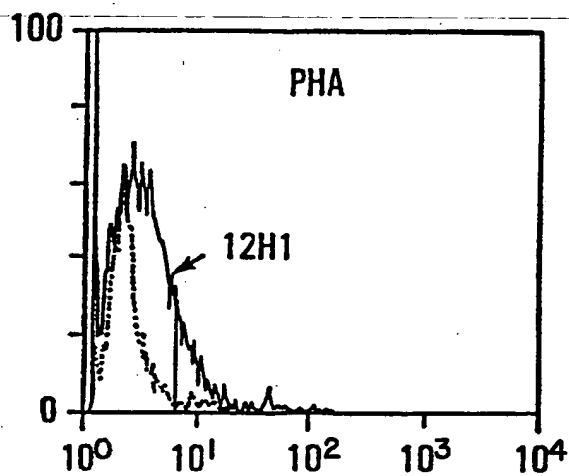


FIG. 2A

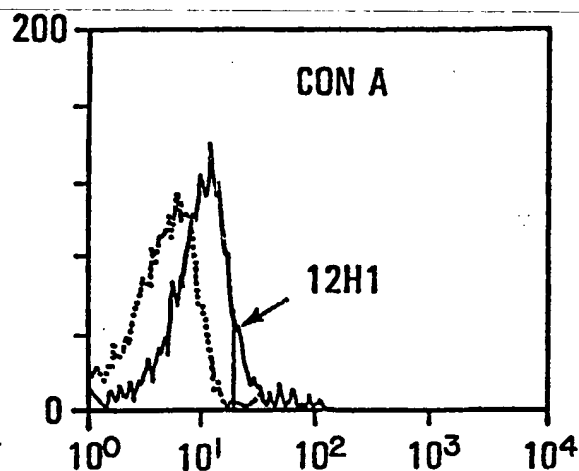


FIG. 2B

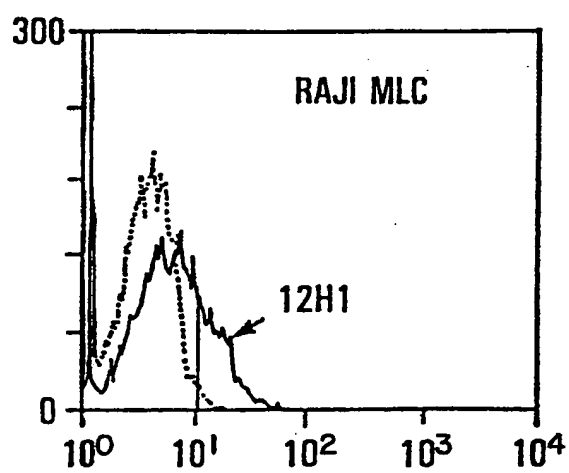


FIG. 2C

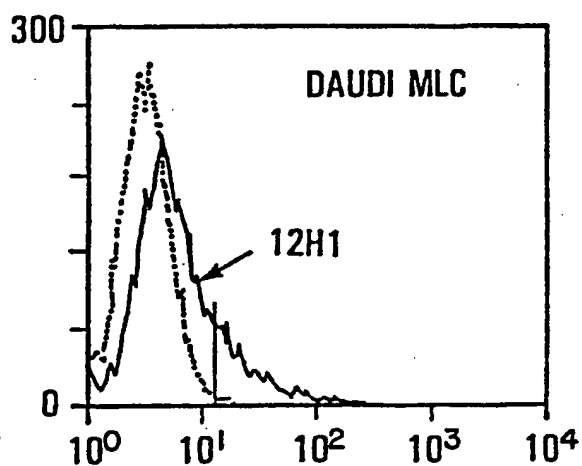


FIG. 2D

3 / 5

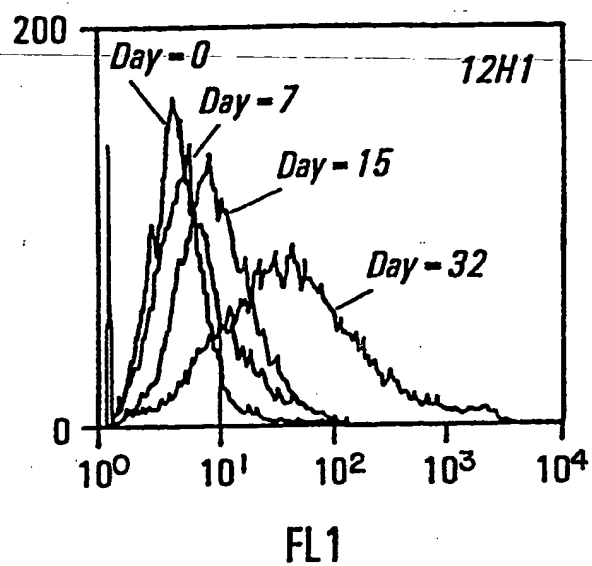


FIG. 3A

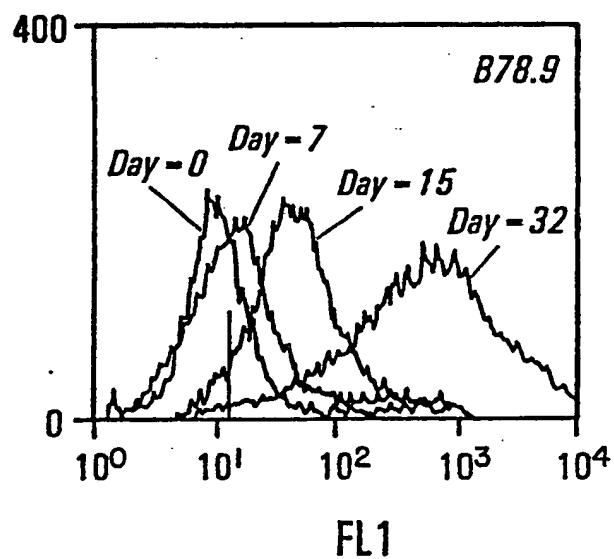


FIG. 3B

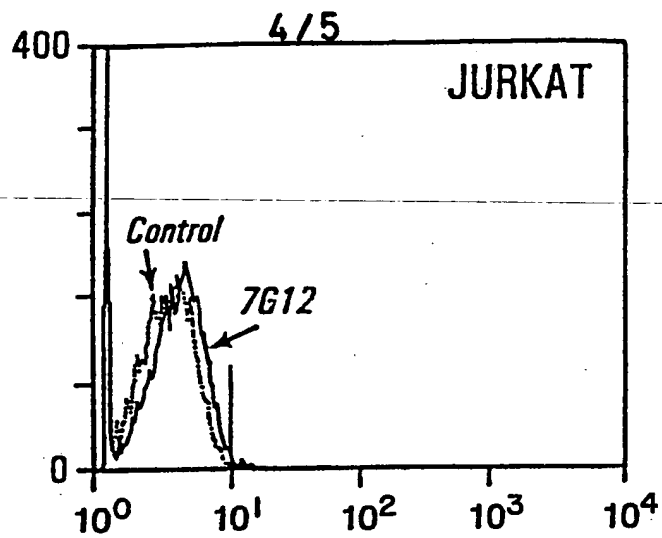


FIG. 4A

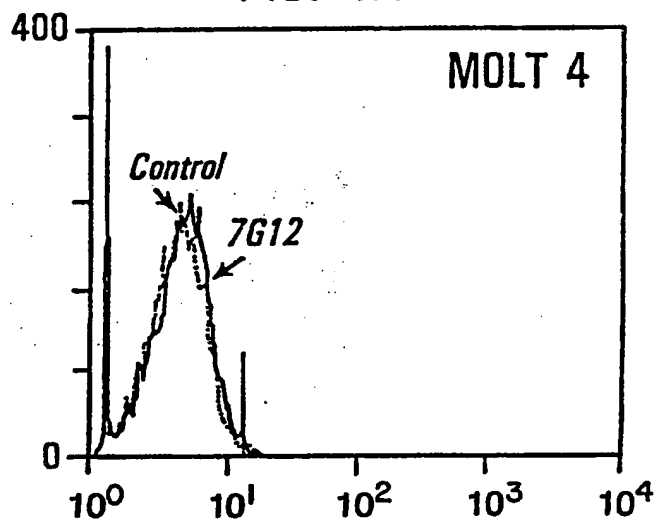


FIG. 4B

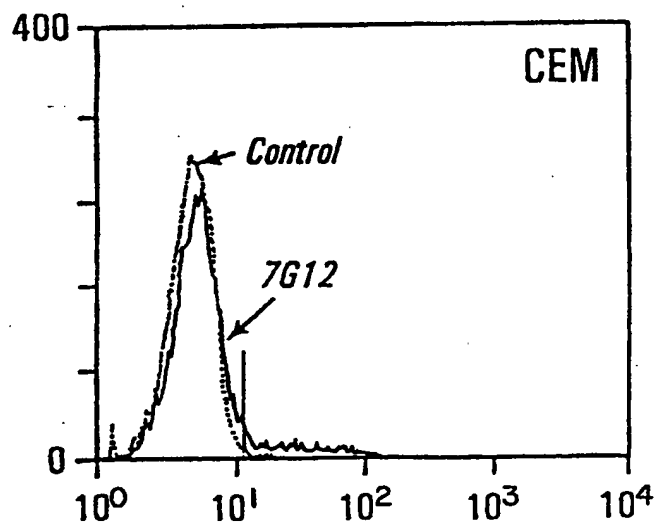


FIG. 4C

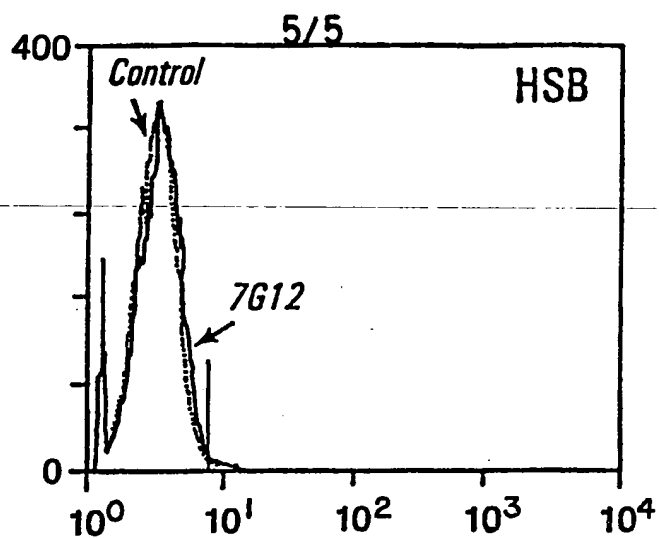


FIG. 4D

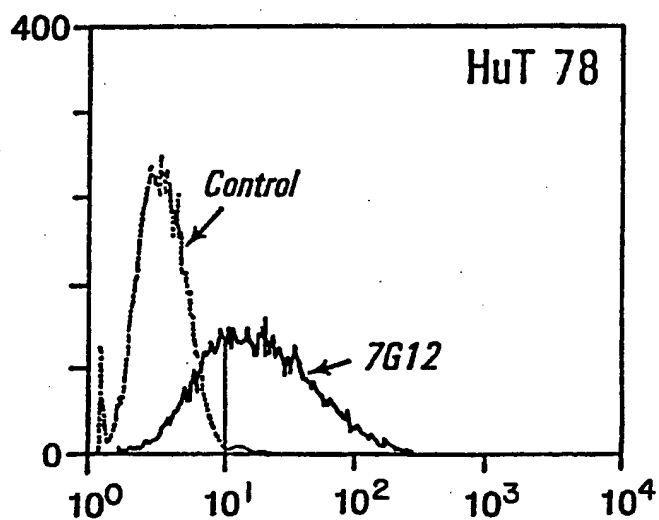


FIG. 4E

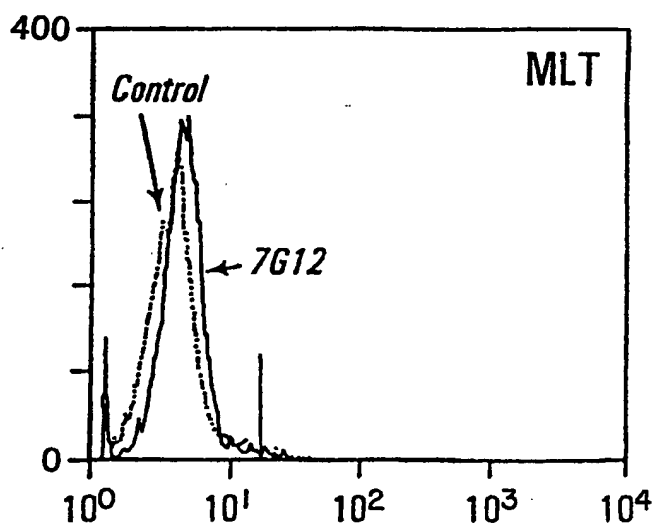


FIG. 4F

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02109

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): C07K 15/00; C07H 15/12; C12N 15/00; G01N 33/53 US CL : 530/350, 387.9; 435/320.1, 7.24; 536/27		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	530/350, 387.9; 435/320.1, 7.24; 536/27	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
CAS ONLINE, MEDLINE, APS, SWISS-PROT, PIR Search terms: proteins, leucocytes, monocytes, receptor, factor V, factor Va, factor X, factor Xa, DNA, antibody, 78 kD		
III. DOCUMENTS CONSIDERED TO BE RELEVANT¹⁴		
Category ⁶	Citation of Document, ¹⁸ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	JOURNAL OF IMMUNOLOGY, Volume 145, No. 1, 01 July 1990, Altieri et al., "Identification of Effector Cell Protease Receptor-1: A Leukocyte-Distributed Receptor for the Serine Protease Factor Xa," pages 246-253, see entire document.	1-20
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 264, No. 5, issued 15 February 1989, Altieri et al., "Sequential Receptor Cascade for Coagulation Proteins on Monocytes," pages 2969-2972, see entire document.	1-20
Y	METHODS IN ENZYMOLOGY, Volume 182, issued 1990, Gerard, "Purification of Glycoproteins," pages 529-539, see pages 529-539.	1-20
Y	METHODS IN ENZYMOLOGY, Volume 182, issued 1990, Ostrove, "Affinity Chromatography. General Methods," pages 357-379, see pages 357-379.	1-20
Y	Lewin, "GENES," published 1987 by John Wiley and Sons (N.Y.), see page 104.	1-20
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²		Date of Mailing of this International Search Report ²
17 JUNE 1992		20 JUN 1992
International Searching Authority ¹		Signature of Authorizing Officer ²⁰
ISA/US		LISA T. BENNETT

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.